Targeted Oncolytic Virotherapy Using Newcastle Disease Virus
Against Prostate Cancer

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Abstract

Prostate cancer (CaP) is the second leading cause of cancer related deaths in men in the United States. Currently, androgen depletion is an essential strategy for CaP combined with surgery, chemotherapy and radiation. Hormone independent cancer stem cells escaping conventional therapy present a major therapeutic challenge. The available treatment regimens for hormone resistant CaP are only palliative and marginally increase survival. Therefore, novel strategies to eradicate CaP including stem cells are imperative. Oncolytic virus (OV) therapy is a novel approach that overcomes the limitations posed by radiation and chemotherapy. Oncolytic virotherapy of cancer is based on the use of replication competent, tumor selective viruses with limited toxicity. Newcastle Disease Virus (NDV), an avian paramyxovirus, is a safe and promising OV successfully used in many clinical trials. NDV is inherently tumor selective and cytotoxic but replication restricted in normal cells. But, systemically delivered NDV fails to reach solid tumors in therapeutic concentrations and also spreads poorly within the tumors due to barriers including complement, innate immunity and extracellular matrix. Overcoming these hurdles is paramount to realize the exceptional oncolytic efficacy of NDV. Therefore, we engineered the fusion (F) glycoprotein of NDV and generated a recombinant NDV (rNDV) cleavable exclusively by prostate specific antigen (PSA). The rNDV replicated efficiently and specifically only in prostate cancer (CaP) cells but failed to replicate in the absence of PSA. Further, PSA-cleavable rNDV caused specific lysis of androgen independent and dependent/responsive CaP cells with a mean effective concentration (EC<sub>50</sub>) ranging from 0.01 to 0.1 multiplicity of infection (MOI). PSA retargeted rNDV efficiently lysed three-dimensional prostaspheres, suggesting efficacy in vivo. Also, PSA-cleavable NDV failed to replicate in chicken embryos, indicating absence of pathogenicity to its natural host, chickens. Prostaspheres generated from DU-145 CaP cell line derived xenografts showed self-renewal, proliferative and clonogenic potential in vitro, and exhibited increased tumorigenicity in vivo. Embryonic stem
and progenitor cell markers like Nanog, Nestin and CD44 were overexpressed in spheres as compared to the cell line suggesting prostaspheres comprise tumor-initiating cells from CaP. Xenograft and cell line derived prostaspheres were permissive for rNDV replication, when the fusion protein was activated by exogenous PSA. The EC₅₀ against tumor initiating cells was 0.11-0.14 MOI, suggesting an excellent therapeutic margin for in vivo studies. PSA retargeting is likely to enhance the therapeutic index of rNDV owing to tumor restricted replication and enhanced fusogenicity. Our results suggest PSA retargeted rNDV selectively replicates and lyse PSA producing CaP cells including tumor-initiating cells and is a promising candidate for immediate Phase I/II clinical trials.
Dedication

I would like to dedicate this work to my wonderful family members, P.S. Raghunath, Geetha Raghunath and Lavanya Raghunath, to my fiancé Sumanth Kumar and friend Padmini Ramachandran for all the love, support and encouragement during my graduate research training at Virginia Tech.
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Attribution

This dissertation is composed of an introduction, conclusion, and three chapters. These chapters are written in journal format, as chapter 2 and 3 will be submitted to journals for publication. The following individuals serve as co-authors on one or more chapters.

Dr. Elankumaran Subbiah, DVM, PhD (Associate Professor of Molecular Virology, VA-MD Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University) is the primary Advisor and Committee Chair. Prof. Elankumaran provided excellent guidance during my research in oncolytic virotherapy of Newcastle disease virus against prostate cancer. Prof. Elankumaran also designed the experiments, provided the resources and helped me in writing the dissertation by providing comments and suggestions. He is the corresponding author for manuscripts from chapters 2 and 3.

Dr. Siba K Samal is the Professor and chair and Associate Dean of Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD 20742, USA. He provided us with the recombinant Newcastle disease virus Beaudette C infectious clone used in this study. He has contributed in writing chapters 2 and 3 and is a Co-author of the manuscripts from chapters 2 and 3.

Dr. Moanaro Biswas is a Postdoctoral Research Associate in Dr. Subbiah’s group. She has contributed to Chapter 3 of the dissertation by guiding the author perform in vitro and mice experiments.

Adria Allen is a co-graduate student in Dr. Subbiah’s group. She was involved in the mice study and data analysis described in chapter 3 of the dissertation. Adria is also a co-author of Chapter 3.
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# List of abbreviations

1. **APMV** | Avian paramyxovirus  
2. **ABC** | ATP binding transporter  
3. **CaP** | Prostate cancer  
4. **CRC** | Cancer repopulating cells  
5. **CSC** | Cancer stem cells  
6. **DAPI** | 4',6-diamidino-2-phenylindole  
7. **EC$_{50}$** | 50% effective concentration  
8. **HSV** | Herpes simplex virus  
9. **HRPC** | Hormone refractory prostate cancer  
10. **IFN** | Interferon  
11. **MOI** | Multiplicity of infection  
12. **MV** | Measles virus  
13. **NDV** | Newcastle disease virus  
14. **OV** | Oncolytic viruses  
15. **PBST** | Phosphate buffered saline tween20  
16. **PSA** | Prostate specific antigen  
17. **rNDV** | Recombinant Newcastle disease virus  
18. **SC** | Stem cells  
19. **SP** | Side population
20. TCID\textsubscript{50}  
50% Tissue culture infective dose

21. TNF\textalpha  
Tumor necrosis factor alpha

22. TDGF1  
Teratocarcinoma derived growth factor one

23. VSV  
Vesicular stomatitis virus

24. VV  
Vaccinia virus

25. Wnt \textbeta  
Wingless integration beta
GENERAL INTRODUCTION

Prostate cancer (CaP) is the second leading cause of cancer related deaths in men in the United States. Currently, androgen depletion is an essential strategy for CaP combined with surgery, chemotherapy and radiation. Hormone independent cancer stem cells escaping conventional therapy present a major therapeutic challenge. The available treatment regimens for hormone resistant CaP are only palliative and marginally increase survival. Therefore, novel strategies to eradicate CaP including stem cells are imperative. Oncolytic virus therapy (OV) is a novel approach that overcomes the limitations posed by radiation and chemotherapy. Oncolytic virotherapy of cancer is based on the use of replication competent, tumor selective viruses with limited toxicity. Among the non-human OV, the avian paramyxovirus, Newcastle disease virus (NDV), is considered to be a promising oncolytic agent (198, 199, 274, 292). Several clinical trials using naturally occurring strains of NDV have been successfully conducted. There is an extensive safety database for NDV, primarily from human tumor vaccine trials and there is no pre-existing immunity in the general human population. Also, NDV infection in humans is always self-limiting without any lasting sequelae or any requirement for therapy or quarantine. There is so far no conclusive evidence for NDV recombination in nature or in vitro (45). Additional characteristics of NDV that supports its use as an oncolytic agent include, absence of a) neurovirulence in humans, b) allergic or asthmatic reactions in humans, and c) the risk of induction of autoimmune disease seems to be minimal. Further, NDV has multiple immune stimulatory properties (30, 175, 351).

However, its immunogenicity and off-targeting to normal cells because of the ubiquitous distribution of sialic acid receptors, are the major concerns in using naturally occurring strains of NDV. The restricted tropism displayed by non-pathogenic and the systemic spread of pathogenic
strains, is primarily due to the fact that the specific tissue proteases required for cleavage activation of the viral fusion (F) glycoprotein and thus for infectivity of progeny are only available in those limited types of tissues. The F protein of virulent NDV strains with multibasic cleavage sites are cleaved by ubiquitous subtilisin-like proteases such as furin, PC6 and PACE 4; whereas, cleavage in non-pathogenic strains with monobasic cleavage site occurs by trypsin-like enzymes found in limited tissues such as tryptase Clara in bronchial epithelium and trypsin-like proteases in the intestines (158, 239, 364).

The unique requirement that the F protein be activated in the target cell provides an opportunity to re-engineer this protein to make its activation dependent on eukaryotic cell surface or intracellular proteases. In particular, it should be possible to exploit the fact that many tumor cells overexpress certain proteases, such as the matrix metalloproteases in many cancer cell types or the prostate-specific antigen (PSA) in prostate cancer cells. The major goal of this proposal was to develop PSA activation mutants of NDV for targeted oncolysis of prostate cancer. As the OV will replicate only in tumor cells expressing PSA, the immune responses to the virus will also be directed only against prostate cancer cells. We hypothesized that

1) Modification of NDV F cleavage site amino acids to a PSA activated peptide sequence will result in PSA specific activation of the mutant fusion protein

2) PSA activated recombinant NDV will specifically lyse prostate cancer cells and enhance its therapeutic potential against prostate cancer

3) Prostaspheres derived from prostate cancer cell lines are enriched for tumor initiating or cancer stem-like cells

4) Oncolytic virotherapy using PSA activated NDV will target cancer stem-like cell population
CHAPTER I

LITERATURE REVIEW

1.1 ONCOLYTIC VIROTHERAPY

Oncolytic virotherapy is a term used for referring to treatment of cancer using a virus. It is also called as oncolytic virus therapy or viral therapy. Since the mid 1900s, there has been a steady trickle of case reports where tumor regression has coincided with natural virus infections (35, 269, 335). Most often the patients in question were suffering from hematological malignancies such as leukemia or lymphoma, known to be associated with significant suppression of immune function. In addition, the remissions were short-lived, typically lasting only for one or two months. In one case, chickenpox led to the regression of lymphatic leukemia in a 4-year-old boy but the remission lasted only one month and his leukemia progressed rapidly until death (35). More recent clinical reports have described the regression of leukemia (108, 260), Hodgkin’s disease (357, 406) and Burkitt’s lymphoma (39) concomitantly with measles infection. Thus, under favorable circumstances, it appeared that certain viruses could destroy tumors without causing undue harm to the patient.

1.1.1 A brief history of oncolytic virotherapy

Virus-mediated tumor regression was most often observed when the patient was young and had a compromised immune system. However, these remissions were generally short-lived and incomplete. Hepatitis viruses were among the first to be used for cancer therapy. As early as 1897, it was noticed that viral hepatitis had ameliorating effects on a variety of human diseases but death due to hepatitis was also reported on
occasions (128, 349). Clinical trials were undertaken in the United Kingdom using glandular fever serum for the treatment of acute leukemia in 1953. In this case, the results were a little more encouraging, as three of five treated patients who acquired symptoms of glandular fever did indeed go into remission (359). Side effects attributed to the treatment were comparatively minor and for a limited duration. Flaviviruses such as West Nile, dengue, and yellow fever were some of the other viruses employed for virotherapy (214, 227-230, 342, 343, 381). The Egypt 101 isolate of West Nile virus was used in more than 150 virus therapy trials against a wide range of cancers (228, 342). Viremia and intratumoral virus replication were confirmed in most patients. Immunosuppressed patients with leukemia or lymphoma were more likely to respond to therapy, but were also at a higher risk of fatal neurotoxicity. These viruses were abandoned as lacking in efficacy or safety and a gradual shift of emphasis turned on to adenoviruses, herpesviruses, paramyxoviruses and poxviruses.

Identified as an oncolytic agent in preclinical models, adenoidal-pharyngeal-conjunctival virus (APC), now known to be an adenovirus) was found to have relatively modest side effects such as inflammation of the eye or pharynx (136). Overcoming this first obstacle, APC rapidly made its way to clinical trials for the treatment of cervical cancer (137). Although APC produced striking effects causing severe hemorrhage and generalized necrosis specifically to the site of the tumor, infection was quickly eradicated by the host immune system and survival was not significantly prolonged. More than half of those who were treated with APC died within a few months after the beginning of the trial. As expected, responses were diminished in patients with pre-existing anti-adenovirus antibodies.
Picornavirus implementation for oncolysis briefly came into vogue thereafter. In one study, published in 1957, tumor xenografts were established by intraperitoneal inoculation of HeLa cells in rats that had first been irradiated and treated with cortisone. Adenoviruses, enteroviruses (Coxsackie A, B, and enteric cytopathic human orphan viruses), vaccinia and vesicular stomatitis virus were tested for intratumoral amplification in this model and Coxsackie B3 virus emerged as the winner (352). Mumps and few other viruses were initially used for metastatic melanoma (217, 252) to stimulate the immune system of those who did not respond to a combinatorial treatment of surgery plus chemotherapy plus BCG vaccine (222). Poliovirus was later used and was shown to cause necrosis and regression in guinea pigs carrying HeLa tumors without any appreciable side effects, (278) but data was still lacking for an oncolytic virus that was efficacious in human trials.

Rodent cancer models were used as in vivo tools to demonstrate conclusively that Russian Far East encephalitis virus could selectively seek out and destroy cancer cells in a living animal, provided the dose was sufficient (227, 229). In the years that followed, many other human pathogens were investigated for oncolytic activity employing rodent models, including Bunyamwera, Ilheus, dengue, yellow fever, West Nile virus (230, 343), and its Egypt 101 isolate (342), Semliki Forest virus, mumps, vaccinia (245), and adenovirus (137). Many of these viruses were also evaluated in clinical trials to demonstrate that complete tumor regression was much more likely to occur in mouse than in the patient (137, 230, 245). This questioned the relevance of the responses seen in hetero-transplanted cancer tissues in the mouse model. Nevertheless, proof of concept in
rodent models quickly became a necessary step to establish oncolytic activity of newly identified oncolytic viruses before clinical testing.

1.2 NATURALLY OCCURRING ANIMAL VIRUSES FOR THERAPY OF HUMAN CANCERS

In an effort to control virulence and at the same time avoid rapid virus clearance resulting from preexisting antiviral immunity, it was hypothesized that a non-human animal virus might retain oncolytic activity even in a host not traditionally susceptible to that particular virus. Two herpesviruses (equine rhinopneumonitis and infectious bovine rhinotracheitis) were identified as oncolytic agents for one or more human tumors (393). Arenaviruses have been little employed as oncolytics after the rather disappointing clinical performance of a virus referred to as the “M-P” virus, now identified as a strain of lymphocytic choriomeningitis virus. It brought about dramatic tumor regressions in rodent models increasing survival by more than 60% (393), yet offered little therapeutic benefit in human clinical trials (224). Although some complete tumor regressions were recorded previously, in nearly every instance, the experimental animal died of infection caused by the virus (227, 228, 230).

Newcastle disease virus (NDV) emerged as an oncolytic virus after it was discovered that intraperitoneal inoculation of NDV in animals with Ehrlich ascites carcinomas was curative with no ill effects that could be attributed to the virus (47). Even upon rechallenge, nearly 90% of mice were found to be immune to subcutaneous injection (48). Naturally occurring strains of NDV were reported to provide remissions lasting at least 10 years and are continuously used in cancer therapy till date (49, 233). However, introduction of wild-type viruses in a traditionally naive host is considered a
risk because these populations have not developed resistance to the virus. Centuries of human exposure to Vesicular stomatitis virus (VSV) infected cattle has led to little more than occasional cases of conjunctivitis, implying it is quite unlikely to cause disease in humans when administered as an oncolytic. VSV, a pathogen of domestic cattle is selectively destructive to human tumor cells with defects in the interferon pathway (350).

Other promising candidates for virotherapy include vaccinia, adeno-, herpes-, reo- and influenza viruses. Studies with influenza virus are in the preclinical stages and it has been shown that the non-structural protein 1 (NS1) inhibits responses to interferon by various mechanisms (234). As expected, the NS1-deletion mutants were strongly attenuated for growth in normal tissues but retained oncolytic activity in interferon-non-responsive tumor cells (258). Vaccinia virus (VV) demonstrated inherent tumor-selectivity and a strong anti-cancer efficacy in clinical trials (18, 139, 154, 217). In a phase I trial, a mutant adeno virus Ad5-delta24-RGD demonstrated clinical efficacy when administered intraperitoneally to patients with recurrent ovarian cancers and was well tolerated (162). Toda et al tested the ICP34.5 mutant HSV-1 strain G207 in a brain metastatic mouse model of human breast cancer and demonstrated prolonged survival (210, 211, 362). However, this effect was found to depend on the cancer cell line that was used in the model, indicating that there may be tumors that are resistant to G207. Hummel et al used an immunocompetent murine model of breast cancer and showed that the ICP0 mutant KM100 completely eradicated tumors in most of the animals treated and significantly increased survival (138). Furthermore, KM100 may have potential as an anti-tumor vaccine since cured mice elicited an anti-tumor immune response and were refractory to subsequent tumor growth. Reovirus (RV) selectively targets cancer cells
with an activated Ras signaling pathway (58, 351). Various phase I and phase II trials using RV (Reolysin®, Oncolytics Biotech) for cancer treatment including breast cancer were performed and it was found to be clinically safe and effective (107, 174).

Russian encephalitis virus had greater oncolytic activity after 20 to 30 passages in sarcoma 180 tumors than the parental strain (230). In 1960, Moore surmised that some progeny of the parental virus could acquire mutations that would be beneficial to virus replication, when propagated in specific cell lines (341). It was then suggested that the alteration of the viral genome could provide improved targeting of oncolytic viruses (341). Martuza et al extended the observation by showing that a thymidine kinase negative HSV replicated in dividing cells but was crippled in non-dividing cells. The thymidine kinase negative HSV was also used to treat malignant gliomas by intracerebral inoculation in mice and was found to prolong survival (142, 212).

Since then recombinant DNA technology is being increasingly used for engineering viruses to enhance their oncolytic potential. The most common strategy was modification of the genetic backbone by insertion of therapeutic transgenes or deletion of virulence genes. Viral targeting to specific tumor tissue types was achieved by virus pseudotyping (for example, addition of integrin αvβ6 binding motifs to adenovirus fibers) (150), using tissue-specific promoters (119), or incorporating microRNA targets into the viruses (79, 392). Further, genes were added to increase the imaging potential of vaccinia viruses, such as the light-emitting vaccinia virus (395), or to sensitize tumors to radioactive iodine via expression of the sodium-iodide symporter (104). Other inserts included the prodrug-converting enzymes or pro-apoptotic proteins such as TRAIL (110, 344). However, the most common method for ‘arming’ oncolytic viruses has been
through the genetic addition of immunomodulatory genes. These included cytokines such as IL-12, IL-18 and co-stimulatory molecules such as CD40L and B7.1 (62). The most common immunomodulatory protein GM-CSF was inserted into adenovirus, herpes simplex virus and vaccinia virus in order to stimulate an inflammatory response within the tumor microenvironment and increase dendritic cell recruitment (153, 159, 205, 330).

Even with the newfound ability to engineer viral genomes, a true therapeutic platform is yet to emerge. The main impediment to using animal viruses for human cancer therapy is the risk of virus evolution giving rise to a new human pathogen that can spread from the patient to other contacts. This risk is not easy to quantify, but it is noteworthy that certain viruses of animal origin like NDV have been administered so frequently to humans, without causing adverse consequences. These animal viruses are now considered to be safe platforms for the development of oncolytics (47-49, 52, 336, 345).

1.3 NEWCASTLE DISEASE VIRUS

Newcastle disease was the name given to a highly pathogenic disease that was first discovered in Newcastle upon Tyne, England in 1926 (200). Deadly infection occurs in over 250 species of birds belonging to 50 orders, both domestic and wild, resulting in substantial losses to the poultry industry worldwide. The causative agent of this disease is a virus, classified in the family Paramyxoviridae, order Mononegavirales and genus Avulavirus (213). Newcastle disease virus (NDV) is also named as avian Paramyxovirus type 1 (APMV-1) (10). The Avulavirus genus contains nine distinct avian paramyxovirus (APMV) serotypes, the evidence of a tenth serotype has recently been reported (11-13, 22, 209, 215, 244, 325, 326, 366, 405). Information about each of these serotypes and the
type of illness associated with them is described in Table 1, adapted from (215). None of the APMV serotypes cause any illness in humans.

**1.3.1 Virus structure**

NDV has a negative sense, single stranded RNA genome which codes for a nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and a RNA directed RNA polymerase (L) protein in the 3’ to 5’ orientation (176). In addition to these gene products, NDV produces two additional proteins, V and W by an RNA editing event that occurs during transcription of the P gene (348). The mRNA editing involves insertion of non-templated G residues and occurs by a polymerase stuttering mechanism (178). The genomic RNA contains a 3’ leader sequence of 55 nucleotides and a 5’ trailer sequence of 114 nucleotides. The leader and trailer sequences flank six structural genes in the order of 3’-NP-P-M-F-HN-L-5’, which encode for at least seven proteins. Leader and trailer sequence are essential for virus transcription and replication (266, 348). At the beginning and end of each gene are conserved transcriptional control sequences known as gene-start (3’-UGCCCAUCU/CU-5’) and gene-end (3’-AAU/CUUUUUU-5’) signals. Between the gene boundaries are intergenic regions, which vary in length from 1 to 47 nucleotides (54, 170). NDV follows the same general mode of transcription and replication as other non-segmented viruses. There is a polar attenuation of transcription such that each of the down-stream gene is transcribed less than its upstream neighbor (236, 266). Thus, the site of insertion of foreign genes determined the level of expression (296). It was also reported that the intergenic sequence (IGS) length modulated transcription of the downstream gene (161). For NDV, foreign gene insertions in the most 3’ locus (upstream
of NP) and the region between P and M genes were well tolerated, stable and did not alter replication kinetics of the recombinant virus (133, 241).

The NP is the most abundant protein in NDV infected cells and in virus particles. It is essential for viral replication and encapsidation of the genomic RNA into an RNase-resistant nucleocapsid (177). The P protein is an essential subunit of the viral RNA-dependent RNA polymerase (177). In addition to playing a role in transcription and replication, P protein acts as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by NP (85). The M protein is peripherally associated with membranes and are seen underlying the viral lipid bilayer (177, 267). The L gene is the last to be transcribed in the viral genome and is the least abundant protein in virus particles (177). The L protein associates with P to form the active viral polymerase and this complex recognizes the helical nucleoprotein complex where NP is tightly associated with genomic RNA (116, 275). Binding of the polymerase complex to the nucleocapsid is mediated by P protein, whereas the catalytic activities are functions of the L protein (67, 127, 275, 328).

The envelope of NDV consists of two transmembrane glycoproteins, HN and F. The HN is a multifunctional protein and is responsible for attachment of the virus to sialic acid containing cell surface receptors. It also possesses neuraminidase (NA) activity that cleaves sialic acid from progeny virus particles to prevent self-aggregation and promotes fusion activity of F protein (307). While HN is a type II integral membrane glycoprotein, F is a type I glycoprotein and is synthesized as an inactive precursor Fo. Fo must be proteolytically cleaved to produce the active fusion protein (307). Cleavage of Fo to F1 and F2 by host cell proteases is required for progeny virions to turn infectious (103, 237). A schematic of NDV particle is shown in figure 1.
1.3.2 Replication cycle of NDV

NDV infection in the host cells depends on the two glycoproteins embedded in the viral lipid membrane, HN and F (99, 176). Initially, NDV infection ensues by attachment of HN to the sialic acid-containing receptors of the target cell (201). The F glycoprotein is activated when the precursor (F0) is proteolytically cleaved at the disulfide link to form F1 and F2 peptides (201, 307). Upon adsorption of HN to its cellular receptors, the interaction between the HN and F proteins at the cell surface triggers a conformational change in the F protein and releases the fusion peptides to fuse the viral and cellular membranes. The stalk region of the HN protein determines the specificity for the homologous F proteins and a L289A mutation in the NDV fusion protein was shown to promote fusion independent of the hemagglutinin neuraminidase protein (186, 187). Viral entry can be either by pH-independent fusion of the viral envelope with the plasma membrane of the host cell or by receptor mediated endocytosis (125). For other paramyxoviruses, it was shown that entry into target cells could also be through a caveolae-mediated endocytosis process (45, 329). After entry, the viral nucleocapsid dissociates from the M protein and is released into the cytoplasm. Subsequently, the polymerase complex transcribes the viral genomic RNA to produce the mRNAs that are required for the synthesis of the viral proteins. The switch from transcription to genome replication takes place when required amounts of viral protein accumulate. The polymerase complex is responsible for the synthesis of full-length plus-strand antigenomic RNA, which in turn serves as the template for synthesis of minus-strand genomic RNA. The nucleocapsid is formed by interactions of the P protein with NP-RNA template and L protein. Viral nucleocapsids are assembled by association of NP
with the newly formed genomic RNA and with the polymerse complex. The assembly and release of infectious NDV particles has been shown to depend on membrane lipid rafts, where HN, F and NP proteins accumulate (175). All components of the virus particle are transported to the plasma membrane where they are assembled under the direction of the M protein. Virions are released from the cell by a process of budding (121). Reports suggest that the M protein is necessary and sufficient alone for NDV budding (257). Finally, the neuraminidase activity of the HN protein facilitates the detachment of the virus from the cell and removes sialic acid residues from progeny virus particles to prevent self-aggregation (178, 355).

1.3.3 Clinical disease

NDV naturally infects via respiratory and alimentary tract mucosal surfaces. Signs of infection with NDV vary greatly from an inapparent infection to severe clinical disease with high mortality depending on factors such as the strain of virus and the health, age and species of the host. The incubation period ranges from 2 to 15 days. An infected bird may exhibit respiratory signs (gaspIng, coughing) nervous signs (depression, inappetence, muscular tremors, drooping wings, twisting of head and neck, circling, complete paralysis), swelling of the tissues around the eyes and neck, greenish, watery diarrhea, misshapen, rough or thin-shelled eggs and reduced egg production. In flocks with good immunity, however, the signs (respiratory and digestive) are mild and progressive and are followed by nervous symptoms, especially twisted necks (213, 285).

1.3.4 Classification of NDV based on virulence

NDV strains display a spectrum of virulence in avian species. NDV can be divided into three different pathotypes based on virulence and severity of disease in
poultry into velogenic, mesogenic and lentogenic. The velogenic strains are further divided into viscerotropic and neurotropic velogenic strains. Viscerotropic velogenic viruses are responsible for acute lethal infections, resulting in hemorrhagic and necrotic lesions in the intestines of dead birds. On the other hand, neurotropic velogenic viruses cause severe respiratory and neurological disease. Mesogenic NDV strains cause respiratory and nervous signs with moderate mortality, while lentogenic NDV strains cause mild infections of the respiratory tract in adult birds and are considered least virulent (9) (10).

1.3.5 Molecular determinants of virulence

NDV virulence is determined by a combinatorial influence of various genetic factors. It has been established that cleavage of the fusion protein precursor (Fo) is the primary determinant of virulence (238, 363). Fo is cleaved into two biologically active disulphide bonded F1-F2 subunits by host cell proteases (178). The amino acid sequence at the F protein cleavage site determines the substrate specificity for different types of cellular proteases (156). Pathogenicity tests in chickens showed that increased number of basic amino acids at the F protein cleavage site resulted in a dramatic increase in virulence of the mutated viruses (256, 268, 298). It has been demonstrated that the HN protein dictates tropism and virulence (69, 135). However, replacement of both F and HN genes of a mesogenic NDV strain by F and HN genes of a velogenic strain did not increase the virulence of the chimeric viruses (86). The individual contributions of internal proteins NP, P and L have also been examined using reverse genetics tools. Some of the studies showed that the NP and P proteins played a minimal role in NDV virulence, whereas, the L protein was a virulence determinant (299). The whole viral
replication complex also reported to contribute towards pathogenicity (76). Finally, the NDV M and V proteins have also been demonstrated to be involved in virulence (8, 134, 259). Proper glycosylation of viral proteins is important for their correct function in the virus life cycle (372). For example, modification of HN’s N-linked glycosylation sites has been shown to decrease NDV virulence (255). Also, addition of nucleotides to the intergenic sequence (IGS) length down-regulated the transcription of the downstream genes and decreased the virulence of mutant viruses in 6-week old chickens (391).

1.3.6 Immune responses to NDV infection

NDV has been shown to stimulate the host immunity to produce cytokines like IFN-α, IFN-β, TNF-α, and IL-1, which in turn lead to the activation of NK cells, macrophages and sensitized T cells (19, 265, 368). Zorn et al (402) showed that cellular cytotoxicity of peripheral blood mononuclear cells (PBMC) enhanced significantly after co-incubation of NDV with effector cells. According to Fournier et al, (94), paracrine stimulation of IFN responses is either through viral HN proteins or viral RNA. Also, virus triggered IFN-α/β is a potent activator of NK cell-mediated cytotoxicity through induction of TNF-related apoptosis inducing ligand (TRAIL) (37, 305). Therefore, activated NK cells are considered as important contributors to innate defense against NDV infection. Besides, IFN-α/β is also important in the generation of CTL activity (373) and cell-mediated cytotoxicity (380).

NDV infection results in potent upregulation of major histocompatibility complex (MHC) class I molecules, antigen recognition molecules (HLA), and cell adhesion molecules (intracellular adhesion molecules (ICAM-I) and lymphocyte function-associated antigen (LFA-3) on the tumor cell surface (112, 379). Moreover, NDV
infection leads to an increased T cell costimulatory activity and consequently, enhanced cytotoxic potential of effector cells (112, 310, 361). Recently, it was reported that dendritic cells with high TLR-3 expression recognized viral double-stranded RNA (dsRNA) leading to maturation and activation (53, 157, 318). Through danger signals, activated dendritic cells promoted cross priming of T cells (20, 182, 380). Activated dendritic cells also increased the expression of costimulatory molecules and stimulated T-cell response (318). Further, NDV administration enhanced the phagocytosis of opsonized erythrocytes by mouse peritoneal macrophages (117). The enhancement of phagocytosis activity correlated with stimulation of nitric oxide synthesis and activation of NF-κB in macrophages (368).

1.4 NDV AS AN ONCOLYTIC AGENT

NDV poses no hazard to human health. Occupational exposure to infected birds (for example in poultry processing plants or laboratory workers) can cause mild conjunctivitis and influenza like symptoms that can be prevented by sanitary measures (55). Interests in its use as an anticancer agent has arisen from the ability of NDV to selectively kill human tumor cells with limited toxicity to normal cells (291). The first report of the application of NDV to treat human cancers was in the early 1960s, when adenovirus and NDV were injected directly into uterine carcinoma resulting in partial necrosis and sloughing (47). Reports also showed the possibility of NDV as a therapeutic agent in cancer treatment from studies both in mouse models and in human clinical trials with favorable results (65, 171). NDV strains have been shown replicate up to 10000 times better in human neoplastically transformed cells than in most normal human cells (265) (314). The selective effect was probably due to the host restriction of V protein and
virus-induced cytokines (IFN-γ and TNF-α) (94) (171). The majority of tumor cells could be infected by NDV and increased viral antigens at the cell surface indicated viral replication (92).

NDV is used to treat human cancer either in the form of free virus or in the form of virus-infected tumor cell vaccine (314). Three cellular mechanisms have been proposed for its anti-neoplastic activities: (i) oncolysis: (336) oncolytic strains may simply kill tumor cells directly (239); (ii) Provision of danger signals: (100, 397) replication of NDV occurs in the cytoplasm of tumor cells. It is associated with the production of single and double stranded viral RNA. These induce danger signals (94) that can be recognized by toll-like receptors (6) in endosomes; and (iii) immune cell activation: NDV may stimulate the host to produce cytokines such as interferons (IFNs) or tumor necrosis factor (TNF), which in turn leads to the activation of natural killer cells, monocytes, macrophages and sensitized T cells (380). To summarize, there are five different conceptual applications of NDV in cancer and disease treatments:

1. For tumor selective cytolysis (oncolysis) (265)
2. NDV as an adjuvant in a tumor vaccine for stimulation of CTL and delayed-type hypersensitivity (DTH) responses after antitumor vaccination (314)
3. For nonspecific immune stimulation and induction of cytokines like interferons (310, 397)
4. NDV as viral vector for delivering therapeutic genes (143)
5. NDV as vaccine vector for immunization against emerging pathogens (74)

1.4.1 Mechanisms of tumor selectivity and tumor cell killing by NDV
As NDV is nonpathogenic in humans and is associated with minor symptoms, several NDV strains are under clinical evaluation for anticancer treatment (129, 265, 336). Also, NDV preferentially replicated in a variety of human tumor cells but not in normal cells (291). The tumor-selective replication of oncolytic RNA viruses such as NDV or vesicular stomatitis virus (VSV) was thought to be due to tumor-specific defects in the cellular antiviral defense. These defects were acquired during the process of tumorigenic transformation and were probably linked to enhanced proliferation and tumor cell survival (350). In normal cells, infection and cytosolic replication of RNA viruses like NDV activated type I interferon (IFN) response leading to the inhibition of viral replication and viral spread. The induction of an antiviral state is maintained by the expression of a variety of IFN-stimulated factors, such as the well-studied intrinsic antiviral enzymes myxovirus resistance proteins (Mx), 2’-5’-oligoadenylate synthetases (OAS) or protein kinase R (PKR) (312). In tumor cells, lowered interferon secretion combined with weak basal IFN expression and impaired induction of IFN induced antiviral proteins were shown to correlate with efficient NDV replication (92, 171, 387). Further, tumor-selective replication of a recombinant NDV, bearing a mutant V protein, was determined by the differential regulation of IFN-alpha and downstream antiviral genes, mostly through the IRF-7 pathway (80).

It was also shown that the oncogenic Ras activity could mediate oncolytic virus susceptibility for viruses like reovirus or VSV by interfering with antiviral responses (246, 351). In addition, efficient translation of VSV depended on tumor-specific defects in translational control (21). For oncolytic NDV, it was observed that N-Ras transformation of human fibroblasts rendered cells a thousand-fold more sensitive to NDV-mediated
cytotoxicity than normal fibroblasts (197). It was recently found that Rac1 is required for oncolytic NDV replication in human cancer cells (283).

The direct mechanism underlying NDV-mediated cytotoxicity is apoptosis induced cell death. Induction of apoptosis requires viral entry, replication, de novo protein synthesis and activation of caspases. Delineation of apoptotic pathways activated in NDV-infected cells revealed common trends, though differences were noted depending on the system used (34, 81, 87, 88, 389). Based on multiple studies, activation of the intrinsic pathway proceeds in a cell-line and virus specific manner and appears to involve the extrinsic apoptotic pathway, ER stress pathway and receptor tyrosine kinase pathways (14, 34, 81, 87, 88, 208, 225, 226, 288-290, 353, 380, 389, 397, 404).

In addition to direct cytopathic effects, anticancer activity of NDV is associated with both innate and adaptive immune response. Infection of murine macrophages with NDV strains La Sota, Ulster and MTH-68/H has been shown to lead to the upregulation of macrophage enzymes such as ADA, iNOS, lysozyme, acid phosphatase, nitric oxide and TNF-α resulting in the increase of in vitro and in vivo antitumor activity (130, 199, 311, 368). In addition, NDV Ulster-stimulated monocytes were shown to mediate tumor cell killing via induction of TRAIL (380). Activation of natural killer (NK) cells has also been implicated in NDV-mediated cytotoxicity against multiple tumor cell lines, with NK cells being the predominant mediator of lysis (402). Infection of tumor cells by NDV leads to expression of the viral HN and F glycoproteins on the surface, which has been shown to change tumor cell surface adhesiveness for erythrocytes and lymphocytes, leading to upregulation of T-cell activation markers in mixed lymphocyte-tumor cell cultures (111). Infection of human tumor cells with NDV Ulster was further shown to
induce production of IFN-β, RANTES and IP-10, and to upregulate the expression of MHC and cell adhesion molecules ICAM-I and LFA-3 (360, 379). These studies show that NDV infection may overcome the inhibitory effect of tumor microenvironment and induce favorable inflammatory antitumor responses.

Tumor-selective replication of rNDV was determined by the differential regulation of IFN-α and downstream antiviral genes induced by IFN-α, especially through the IRF-7 pathway. In a nude mouse model of human fibrosarcoma, the IFN-sensitive NDV variants were as effective as IFN-resistant rBC virus in clearing the tumor burden. In addition, mice treated with rNDV exhibited no signs of toxicity to the viruses (80).

1.4.2. Oncolytic virotherapy using natural NDV strains

There are several naturally occurring NDV strains with varying virulence that have shown efficacy in preclinical models (5, 24, 47, 165, 197-199, 273, 291, 314, 367, 402). Several of them are being used in human clinical trials. They include, strain 73-T, MTH-68, PV-701, Ulster and HUJ.

NDV lytic strain 73-T replicated in human tumor cells such as fibrosarcoma, osteosarcoma, neuroblastoma, cervical carcinoma and Wilm’s tumor causing cell-cell fusion, syncytium formation and cell death (197, 291). In addition, the oncolytic potency of NDV strain 73-T was demonstrated in mice with human tumor xenograft models. Intratumoral and intraperitoneal injection of NDV strain 73-T caused complete tumor regression in athymic mice bearing human neuroblastomas and fibrosarcoma xenografts (197). Another study showed locally administered virus was able to inhibit tumor growth (77 to 96%) in several carcinoma xenografts in mice including cancer of epidermoid,
colon, lung, breast, and prostate xenografts (273). Furthermore, complete tumor regression was observed in 9 out of 12 mice bearing IMR-32 neuroblastoma tumor xenografts after a single intraperitoneal injection of NDV with no adverse effect on normal human cells (197).

The first clinical documentation of NDV 73-T activity involved one cervical cancer patient. In this report, $2.4 \times 10^{12}$ virus particles were injected directly into the tumor demonstrating intratumoral regression of the local cancer and also a distant malignant lymph node (402). Partial necrosis and sloughing were observed but this was followed by tumor regrowth.

Viral oncolysate preparations (lytic strains, Italien and 73-T) containing plasma membrane fragments from NDV-infected cancer cells were also used as anticancer vaccines. Phase II clinical trials using viral oncolysates were performed on patients with malignant melanoma (233). In this study, 6 out of 13 patients showed a decrease in the size of the skin nodules and/or lymph node lesions. In another trial, 83 patients at stage II malignant melanoma were treated with NDV oncolysates as an immunotherapeutic agent in post-surgical management (49). The patients were observed for at least 10 years and more than 60% were free of recurrent disease. Continued monitoring of the patients showed a 55% of overall fifteen-year disease-free survival (DFS). Extended survival was observed among patients who displayed an increase in the number of CD8+ CD56+ T lymphocytes that induced production of large amounts of cytokines like TNF-α and IFN-γ to aid in cytotoxicity (29).

To prepare the autologous ATV-NDV vaccine, a nonlytic, avirulent strain Ulster was used in the culture of tumor cells derived from patients. There are a lot of successful
test cases in preclinical and clinical studies, without any severe adverse effects (49, 51, 197, 315). This strain has a monocyclic abortive replication cycle in tumor cells (314). Initially, the virus is adsorbed on to the tumor cells for an hour to bind efficiently. It remains in the body for generation of effective immune responses, mostly T-cell-mediated immunity. Direct contact of virus with immune cells will affect cell proliferation and activation status. As viral replication takes about 10 to 50 hours in tumor cells, it is sufficient for the generation of DTH skin responses (314). Clinically, the ATV-NDV was tested in 23 patients with colorectal liver metastases and vaccination was applied to the patients after they underwent liver resection (189). As a result, the patients showed increased recurrence and DTH skin reactivity. In another study, favorable results were obtained when a dose of $1 \times 10^7$ human colorectal tumor cells were subcutaneously administered together with 32 hemagglutination units (HAU) of NDV to colorectal cancer patients (40). After four vaccinations at two-week intervals, the DTH responses were increased at distant sites. This indicated an augmentation of tumor reactive T lymphocytes.

ATV-NDV was also used as a surgical adjuvant vaccine for 208 patients with locally advanced renal cell cancer. Vaccination caused a median DFS of 21 months that was higher than the historical controls (163). With a two-year follow-up, the analysis showed 10 relapses (18%) among the patients along with a median follow-up of 39 months. Besides, encouraging results were obtained in a nonrandomized study involving 23 glioblastoma patients vaccinated with ATV-NDV (163). The results showed that vaccination lead to improvement of the median DFS significantly higher than the control group. It was proved that the quality of the ATV-NDV vaccine is critical for antitumor
efficacy (309). Hence, improvement of the quality and efficacy of ATV-NDV vaccine was carried out by modifying the vaccine preparation step by enrichment of the tumor cells through Percoll centrifugation followed by the removal of tumor-infiltrating leukocytes (349) using immunomagnetic beads (250). The improvement of antitumor efficiency of ATV-NDV vaccine could also be achieved by the addition of recombinant IL-2 (152, 277). Vaccinated patients benefited with improved survival rate with three-year and five-year survival rates of 67% and 61%, respectively (152). In another study, the antitumor efficiency of ATV-NDV was enhanced with the aid of recombinant bispecific hybrid antibodies (112). The antibody coated ATV-NDV caused upregulation of T cell activation markers (CD3 and CD28) within 24 hours. In summary, the ATV-NDV vaccine appeared to be feasible and safe to treat advanced cancers such as colorectal cancer, breast cancer, ovarian cancer, glioblastoma, kidney cancer, and head and neck cancer (4, 40, 152, 309, 347).

First reports of the oncolytic potential of strain PV701 was by Lorence et al. (196). Intravenous administration of PV701 in a dose escalation study in tumor bearing mice produced partial tumor regressions at doses as low as $6 \times 10^5$ plaque forming unit (pfu). More than 80% of the mice developed complete tumor regressions at doses up to $6 \times 10^8$ pfu. The antitumor response was associated with evidence of viral replication. These encouraging results led to initiation of a phase I clinical trial to intravenously administer PV701 strain to advanced solid cancer patients. Intravenous administration of NDV strain PV701 vaccine was performed on 79 patients with solid tumors (265). In this study, the virus strain caused regression of advanced solid cancers without observed cumulative toxicity. One patient’s squamous cell cancer on his tonsil was completely eliminated after
NDV therapy. Measurable tumor reductions were seen in seven other patients with diverse malignancies (265). Unfortunately, one possibly treatment related death occurred. Post mortem revealed inflammation in the lungs suggesting rapid tumor lysis leading to compromised pulmonary function after therapy. NDV strain PV-701 was administered at an intravenous dosage of at least $3 \times 10^9$ infectious units and at least $4 \times 10^{12}$ infectious units by intratumoral route (129). The adverse effects were dose-dependent and included flu-like symptoms, leucopenia and neutropenia. Occasionally, virus infection was associated with transient thrombocytopenia and diffuse vascular leakage (265). Presence of viral particles was observed in the tumor tissue of treated patients but not in the heart, lung, kidney, liver, or brain tissue (265).

First reports of strain **Hertfordshire, a velogenic, viscerotropic strain**, for cancer treatment was by Csatary (63). He developed a novel virus strain named **MTH-68/H**, which meant “More Than Hope 1968”. MTH-68/H strain has the ability to cause significant regression of human tumors from cell lines such as PC12, MCF-7, HCT116, DU-145, HT-29, A431, HeLa, and PC3 cells. MTH-68/H is also the most potent IFN-α inducer among all NDV strains tested (17). It has the ability to induce nitric oxide (NO) and to increase the macrophage population in treated rats resulting in enhancement of antitumor effects (130). In a Phase I clinical trial, MTH-68 strain was administrated to 33 patients with advanced cancers twice weekly (65). Favorable responses were observed in a total of 18 patients (55 %) compared to 2 patients in the placebo group (only 8%) (65). Treatment of liver metastases of luciferase transfected murine CT26 colon carcinoma cells with NDV strain MTH68/H resulted in a significant delay in tumor growth and prolonged survival without severe side effects. Loss of body weight did not occur among
the vaccinated mice (17).

NDV strain **HUJ** (OV001) is a lentogenic and a highly purified isolate originally derived from naturally attenuated B1 NDV vaccine strain. This strain has high selective cytopathogenicity to human and animal cancer cell lines. The virus progenies produced by the lentogenic strain are noninfectious because of incomplete processing of the fusion (F) protein. Besides direct cytotoxic effects on target cells, HUJ also induces cytokine-mediated events and augments the immune reactions (95).

In a recent phase I/II human clinical trial, NDV strain **HUJ** was administered intravenously (11 billion infectious units) to 11 patients with recurrent glioblastoma multiforme (GBM). One patient experienced stable disease after the first cycle of vaccination and later complete tumor remission with duration of 3 months. Intravenous administration of NDV strain HUJ vaccine is well tolerated. The encouraging responses of strain HUJ warrant the evaluation of NDV in other cancers, besides GBM (95).

Another lentogenic strain, **LaSota** was also shown to induce antitumor cytotoxic effects of mouse macrophages by the production of TNF-α (380). The anticancer activity of activated monocytes was attributed to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (380). In the study by Liang et al. (188), vaccination of NDV strain LaSota was efficient to prolong colorectal cancer patient’s life with 96% patient survival after one year treatment with NDV immunotherapy.

Intratumoral injection of highly virulent and velogenic NDV strain **Italien** displayed high sensitivity to human metastatic melanoma xenotransplants in nude mice (313), with a maximum tolerated dose (MTD) in the range of 2000 hemagglutinating units (33). However, the nonlytic strain Ulster showed stronger cytotoxicity effects on a
CT26 colon carcinoma model (313). This suggested that the antitumor effect on different tumor cell types correlated with NDV virulence. Importantly, NDV virus replication did not happen in normal cells, including resting T lymphocytes and normal chicken liver cells (314).

Table 2 shows the list of NDV strains used in clinical trials (adapted from the National Cancer Institute at the National Institute for Health website).

In view of the evidence accumulated to date, no conclusions could be drawn about the effectiveness of using NDV in the treatment of cancer. Most reported clinical studies involved few patients and historical control subjects rather than actual control groups. Poor descriptions of study design and incomplete reporting of clinical data has hindered evaluation of many of the reported findings. However, according to National Cancer Institute’s summary of evidence for NDV, “while most studies are small and lack adequate controls, the number of studies suggesting a potential clinical value warrants further action”.

### 1.4.3 Advances in virotherapy

Initially oncolytic viruses were programmed to replicate in tumor cells by targeting cellular signaling activities (173), incorporating a therapeutic transgene that targets tumorigenic pathways (194) or retargeting to cellular receptors that are essential for signaling (such as epidermal growth factor receptor) (15). It was possible to develop an oncolytic adeno virus that replicated specifically in prostate cancer cells. Viral replication could be controlled by the expression of prostate-specific antigen (PSA) regulated genes (394). Alternatively, deletion of the adenovirus E1B gene created a virus that could replicate exclusively within cancer cells because of aberrations in nuclear mRNA export,
a common phenomenon occurring in most cancer cells (248, 249). It was further reported that adenovirus induced ERK activation was critical for viral replication (320).

Attempts were made to engineer other viruses such as herpes, VSV and vaccinia by targeting cell signaling pathways. A HSV g34.5 deleted mutant showed enhanced replication in cells with activated mitogen-activated protein (MAP) kinase or extracellular signal-regulated (103) kinase. This in turn inhibited protein kinase R activity thus circumventing the negative impact of the IFN signaling pathway (339, 371). Similarly, viruses such as VSV showed preferential replication in cells with an activated Ras-ERK pathway and defective IFN pathways (246). A vaccinia virus mutant with a deletion in B18R also showed IFN-dependent cancer selectivity and efficacy (166).

As cell surface recognition and virus entry is the key first step to a productive viral infection, engineering a virus to only recognize the tumor cell surface would restrict replication of a potent oncolytic virus to malignant cells. During tumor evolution, various genetic and epigenetic events led to the unique display or overexpression of tumor antigens on the surface of malignant cells. Measles virus binds by its haemagglutinin (H) attachment protein to one of two cellular receptors: CD46, a member of the complement regulatory protein family (75, 242) and SLAM (signalling lymphocytic-activation molecule) (131, 358). Initial targeting strategies modified the measles H-protein with carboxyl terminal extensions that included domains from growth factors such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF1) (317), as well as from single-chain antibodies (scFvs) against tumor antigens such as carcinoembryonic antigen (CEA) (118), CD20 (43) and CD38 (270). These attachments provided the virus with some level of tumor targeting in cell culture and xenograft models but a large
amount of virus was still found to bind to the normal cell antigens CD46 and SLAM. This raised problems in using measles virus as a therapeutic vector as infection of normal cells through SLAM is known to mediate transient immunosuppression (114).

An alternative approach is to use the *in vivo* tumor environment to augment selectivity. For example, subtle alterations in the fusion (F) protein of measles virus allow it to be processed to an active form only in the protease rich tumor microenvironment (203). The F protein of measles virus facilitates viral entry into cells by mediating fusion of the viral and cellular membranes and is normally produced as an inactive precursor that is naturally cleaved by furin to expose a fusion peptide. As many cancer cells secrete high levels of proteases, such as matrix metalloproteinase 2 (MMP2), it was reasoned that a virus that produced an F protein with the furin site replaced by an MMP2 cleavage site (FMMP2) would be preferentially activated in the vicinity of tumor cells. Indeed, when FMMP2 is transfected into HT1080 cells, multinucleated syncytia were formed indicating that the fusion protein was activated by MMP2 (346). In nude mice models, the MMP-activated MV retarded HT1080 xenograft growth as efficiently as the furin-activated MV vaccine strain. Also, in MV-susceptible mice, the furin-activated virus caused lethal encephalitis upon intracerebral inoculation, whereas the MMP-activated virus was non pathogenic (346). Thus, the advantage of using viruses that can be activated by components of the tumor microenvironment is targeting. These viruses can be tailored to infect specific types of cancer cells depending on the types of proteases secreted.

1.4.4 Genetic manipulation of NDV to enhance its therapeutic potential as an oncolytic virus

The therapeutic index (also known as therapeutic ratio) is a comparison of the
amount of a therapeutic agent that causes the therapeutic effect to the amount that causes death (in animal studies) or toxicity (in human studies). Enhancing the therapeutic index of an oncolytic virus can be achieved by genetically tailoring the virus to infect and replicate specifically in cancer cells, thereby reducing off-target losses of the virus.

NDV is inherently oncolytic and therefore, by employing various strategies, the therapeutic index of NDV can be enhanced. Introduction of the IL-2 gene into the viral genome increased its immunostimulatory properties (1, 144). Significant tumor regression and T-cell infiltration were observed when human colon carcinoma tumor bearing mice were treated with recombinant NDV-IL-2 (144). NDV-IL-2 oncolysate also resulted in activation of tumor-specific CTL and memory T cells (144). Janke et al. inserted a recombinant granulocyte monocyte colony-stimulating factor (GM-CSF) as an additional transcriptional unit into NDV (143). A recombinant NDV strain (B1) expressing the influenza NS1 gene led to a high degree of T-cell infiltration, suggesting tumor specific CTL response in mice (396). Another recombinant NDV strain was designed, in which the virus was modified by pre-incubation with a recombinant bispecific protein (IL-2 receptor) (33). A new binding site was introduced on the virus that enhanced its interaction to tumor-associated target. Higher virus replication efficiency was noticed in the Eb-M7 (IL-2 receptor positive) syngenic tumor bearing mice (33). Administration of modified NDV revealed that side effects were reduced without affecting the antitumor activity in this model.

The presumed specificity of NDV for cancer cells is thought to be due to defective innate immune responses in some cancer cells. An attenuated rNDV of the mesogenic Beaudette C strain with a mutation in the V protein was generated. The resultant virus
was attenuated in its ability to replicate in primary cells and tumor cell lines possessing intact IFN responses, but was equally effective to the parental virus in tumor cell lines and xenografts possessing defective components of the innate immune response (80). In conclusion, genetically modified NDV strains may have not only antitumor effects but also augment immunomodulatory effects.

1.4.5 Safety and efficacy of NDV as oncolytic agent

The tumor selectivity of NDV makes it an attractive oncolytic virus (243). NDV strains can selectively replicate up to 10000 times better in tumor cells than in normal cells (291). Numerous reports have shown that the virus does not replicate in non-transformed cells such as fibroblast cells, resting T lymphocytes and normal human primary cells (314) (291) (197) (87). Besides, NDV is an immunostimulatory agent as it can induce antitumor activities of a variety of effector cells including NK cells, macrophages and CTL (311, 373, 380, 397).

Several studies documented NDV to be well tolerated in relatively high doses and with minimal adverse events in human cancer patients. According to Pecora et al. (265), oncolytic NDV strain is well tolerated in doses of at least $3 \times 10^9$ infectious units by the intravenous route and at least $4 \times 10^{12}$ infectious units by the intratumoral route (129, 265). There is no report on accumulative toxicity associated with repeated administrations of NDV as evidenced in one cancer patient who received over 30 courses of PV701 without any adverse events (265). Ockert et al. had reported the five-year survival benefits in phase II trials using ATV-NDV (using a non-virulent strain Ulster) that involved patients with locally advanced colorectal carcinoma (250). Another study with the ATV-NDV revealed 61% of vaccinated patients with stage III and stage IV head
and neck squamous cell carcinoma experienced increase of five-year survival rates (152).

The general adverse effects displayed on vaccinated patients were conjunctivitis, laryngitis, hypotension, and mild flu-like symptoms, including fever, chills, tiredness, headache, muscle pain, and weakness (250, 309, 319). Erythema, swelling, induration, and itching were observed on the vaccination sites (250, 319). Other typical side effects observed in some patients were leucopenia and neutropenia. Occasionally, virus infection was associated with transient thrombocytopenia and diffuse vascular leakage (265). These side effects were temporary and disappeared in 1 to 2 days post vaccination.

In conclusion, the safety of NDV strains as anticancer agent has been consistently high with low toxicity (243).

1.5 THE ROLE OF STEM CELLS IN PROSTATE CANCER DEVELOPMENT

Cancer stem cell hypothesis focuses on three cell types for tumor progression and recurrence. The first is the normal stem cell, the second is the cancer cell of origin and the third is the cancer-repopulating cell (CRC). Cancer stem cells (CSCs) have now been reported in multiple solid tumors including cancers in the breast, brain, colon and pancreas (7, 184, 334). Conventionally, stem cells were defined by their ability to self-renew and differentiate into multiple lineages (280). The cancer cell of origin is not necessarily a stem cell in normal tissue but may also be a progenitor cell, which is susceptible to malignant transformation. The CRC is defined as a population of biologically distinct tumor cells possessing stem cell properties. These cells also possess the ability to self-renew, repopulate the tumor after chemotherapy and have been reported to play a role in subsequent metastasis (41, 293, 386).

1.5.1 Prostate cancer
Prostate cancer (CaP) is the second leading cause of cancer related deaths in the United States (145) and its incidence is increasing in many countries including Japan. Radical prostatectomy is the most common treatment for organ confined or localized prostate cancer in the United States. This and radiation therapy resulted in an overall rate of survival of 5-10 years post-treatment. Hormonal treatment also referred to as androgen deprivation (depriving the prostate of testosterone) is also used to treat patients with localized CaP. However, the persistence of castration-resistant cells within prostate tumors and their ability to proliferate in the absence of androgen causes the lethal hormone-refractory phase of prostate cancer (308). Most of the patients that received androgen ablation therapy showed an initial response, but eventually developed androgen-independent prostate cancer within 12 to 18 months of androgen deprival. Chemotherapeutic agents and anticancer drugs such as docetaxel are used for treating hormone resistant prostate cancer and are palliative (169). Although clinical experience with the FDA approved prostate cancer vaccine, sipuleucel-T (Provenge) is limited; it has also shown to improve only survival. Unfortunately, second-line treatment options are limited and most patients presented with metastases perish within 5 years of diagnosis (168). There is currently no effective cure for advanced and metastatic CaP and novel therapeutic strategies are essential.

1.5.2 Normal prostate stem/ progenitor cells

Two fundamental traits of stem cells (SCs), self-renewal and multi-potency were first established in studies of hemopoiesis (185, 383). Evidence that strongly supported the existence of SCs in the human prostate was from the classic androgen cycling experiments (83). Observation that the adult rodent prostate can undergo multiple rounds
of castration-induced regression and testosterone induced regeneration indicated that a small population of cells possessed the ability to both self-renew and differentiate while the bulk androgen dependent terminally differentiated cells lacked such an ability (83, 180, 383).

*In vitro* sphere-forming assays were developed to study primitive cells from the prostate (102, 106, 181, 323). A sub-fraction of the human and mouse prostate basal compartment could generate spheres that self renewed in a three-dimensional semisolid structure comprised of extracellular matrix components (106). In the prostate sphere assay, primitive cells retained their ability to generate daughter spheres for more than 10 successive passages and a subset of sphere cells retained the capacity to generate prostatic tubules when transplanted *in vivo* (390). A greater number of cells had sphere-forming activity than prostate regenerating activity, suggesting that the sphere assay measures both progenitor cell and stem cell function. Using markers first identified in the mouse prostate, it was found that cells from the basal fraction exhibit robust tissue-regenerative activity when transplanted into immunodeficient mice and the dissociated cells from primary outgrowths demonstrated cellular heterogeneity (105).

Several candidate populations of prostate stem/progenitor cells have been reported including those expressing high levels of CD44, integrin α2β1 or CD133 (356). CD44 is expressed on most basal cells and has been implicated in tumor metastasis (262, 264, 294). The experimental evidence that most of the cells that survived castration were basal rather than luminal cells, led to the traditional hypothesis that the basal-cell layer harbors self-renewing SCs (83, 180). Some of the key molecules that normally regulated SC self-renewal and survival, like p63, hTERT, CD133, CD44 and Bcl-2 were preferentially
localized in the basal layer (31, 190, 216, 356, 364). Further, basal cells have also been shown to differentiate into luminal cells (180, 190).

Although reports support a basal location for stem cells in the prostate, it does not necessarily imply that all luminal secretory cells were terminally differentiated. Several groups had investigated the possibility that luminal cells with stem/progenitor characteristics existed in the rodent prostate. While basal cells preferentially survived androgen ablation, a subset of luminal cells demonstrated castration-resistance as well (83, 364). It was also shown that in the castrated mouse prostate, a population of castration resistant luminal cells expressing the homeobox transcription factor Nkx3-1 (CARNs) could generate prostate tissue with basal, luminal and neuroendocrine cells (376).

An important issue under debate is about the cell-of-origin for prostate cancer (132). Several reports suggest primitive cells as efficient targets for transformation (26, 261, 340, 401), although evidence also exist to support a role for mature cells in oncogenesis (61, 140, 172).

1.5.3 Prostate cancer stem cells

It was reported several decades ago that only a minor subset (0.01–1%) of cancer cells isolated from tumors had the ability to regenerate a clonal growth or a tumor (293). Pioneering work by John Dick and Bonnet (41) on acute myeloid leukemia provided the first direct evidence for CSCs that was later reported in many solid tumors (7, 184, 334). It was shown that a sub-fraction of cancer cells could propagate the tumor in mice, while the remaining fraction was depleted for this activity (7, 41, 179, 247, 334). However, multiple groups reported notable exceptions where the majority of primary cancer cells
had tumor-propagating potential (158, 284, 388).

Originally, a hierarchical model of CRC differentiation suggested that CRCs and their progenitors gave rise to more differentiated cells with less regenerative potential (72, 293). This model was based on fractionation of tumor cells using cell surface markers to isolate rare subsets of tumor cells, which displayed exclusive tumor regenerating potential in colony-forming assays and \textit{in vivo} transplantations in immunodeficient mice (41, 247). Over the last decade, significant amount of work was performed to establish xenografts of primary human prostate cancers. Numerous groups had demonstrated that small bits of cancerous prostate tissue isolated from both primary and metastatic sites could be propagated and serially passaged in mice (59, 82, 167, 274, 281, 282, 374). Several other groups used cell lines and xenografts as models to investigate potential prostate cancer stem cells (141, 262, 264, 369). Morrison and colleagues demonstrated that optimization of assay conditions vastly altered the number of cells capable of tumor formation in mice (284).

Collins and Maitland suggested that putative human prostate epithelial SCs bear the CD44+$\alpha_2\beta_1$ hiCD133+ phenotype (294). Different research groups reported that prostate CSC fractions could be successfully enriched by some cell surface phenotypes, especially CD44, CD133, $\alpha_2\beta_1$ integrin, TRA-I60, CD151, CD166 and CD117 (192, 193, 204, 286). Although these proteins were known to be involved in embryonic and somatic stem cell function of hematopoietic system (272), their functional role in the development of CaP CSCs remains undetermined. Huang \textit{et al.} studied the relationship between the SC marker CD44 and neuroendocrine (NE) cells and found that most NE cells also expressed CD44 (253). On the other hand, as a functional approach, non-adherent sphere culture is
being widely employed for enriching the potential CSC subpopulations in vitro (89, 98, 279, 287, 333).

1.5.4 Role of cancer stem cells in tumor dormancy and metastasis

Tumor dormancy has long been recognized as a cause of metastasis in breast and prostate tumors and metastasis can occur many years after treatment (91, 185). In principle, CSCs that are endowed with the self-renewal ability could founder a colony in a distant site while differentiated cells that generally lack the self renewal capacity would not proliferate well to establish a metastatic colony (91, 365). Epithelial-mesenchymal transition (EMT) plays an important role in metastasis and a recent study demonstrates that breast cancer cells induced to undergo EMT also acquires CSC traits (207). CSCs may stay quiescent and switch from being dormant to proliferative due to environmental changes giving rise to recurrence and metastasis. Also, they are generally more resistant to chemical and radiation therapies making them the lethal seeds for metastasis (27, 90, 91). While CD133 is a widely used CSC marker, it was reported that the CD133+ cell population alone could not produce metastasis in an orthotopic pancreatic cancer model but the combined CD133+CXCR4+ subpopulation showed strong metastasis (124). Further, the CD133- colon cancer cells have been shown to be even more aggressive and metastatic than their CD133+ counterparts although both populations could initiate tumor development (324).

In benign prostatic hyperplasia, CD133+ cells expressed genes relating to undifferentiated cells such as TDGF1 and targets of the Wnt and Hedgehog developmental pathways. CD133- cells, on the other hand, showed upregulated proliferation and metabolism genes related to a transient amplifying population (322).
Array analysis on cultured samples of localized primary human prostate cancer revealed that CD133+ cells displayed a proinflammatory phenotype by increasing NFκB expression (36). These findings suggest that metastasis and tumor initiation might be mediated by distinct cancer cell populations (123) and there might also exist metastatic CSCs (218, 382). In summary, the true interrelationship between CSCs and metastasis awaits more in-depth studies and it is anticipated that novel therapeutics that specifically target CSCs may prevent metastasis.

1.6 SIGNALING PATHWAYS IN PROSTATE CANCER STEM CELLS

By definition, both normal and CSCs must maintain self-renewal capacity and give rise to differentiated progeny cells. Thus, it is possible that the pathways governing these processes may be similar between them. It is now well known that tumor growth is determined by disregulation of the signaling pathways and molecular mechanisms regulating cell proliferation, differentiation and self-renewal (195).

1.6.1 Wnt/β-Catenin Pathway

Wnt signaling defects are associated with several tumor types including colon, skin, breast, prostate and bone marrow (28, 42, 223, 292, 354). Wnts are a 19-member family of secreted glycoproteins that bind to several different cell surface receptors and determine signal transduction by the canonical or non-canonical pathway (44). The Wnt pathway is associated with the development of bone metastases after the onset of prostate cancer (115). Two independent studies demonstrated increased β-catenin gene expression in cell line derived CD44+ CSCs relative to the non-stem cell population (141, 262). Wnt activity regulates the self-renewal of prostate cancer cells with stem cell characteristics independent of AR activity (38). Inhibition of Wnt signaling may therefore have the
potential to reduce uncontrolled prostate cancer stem cell renewal.

1.6.2 Hedgehog signaling Pathway

Normal mammalian prostate development requires functional Hedgehog (Hh) signaling (30, 96). In mature prostate cells, Hh expression is low (400), which indicates its requirement only during development and differentiation steps involving stem and transit amplifying cells. In addition, Hh glycoprotein expression increases in prostate cancer cells (16, 30, 303). It was shown that SMO (smoothened G-protein-coupled-receptor) mRNA was highly expressed in xenograft tumors initiated from CD44+ stem-like cell subpopulation in DU145, LAPC4 and LAPC9 human prostate cancer cell lines relative to the CD44- cells (262). These findings support the notion that Hh pathway is a key component in CSC maintenance, as injection of Hh pathway inhibitors like cyclopamine completely regressed xenografted tumors in mice (30).

1.6.3 Notch signaling Pathway

The Notch pathway is linked to both oncogenic effects and tumor suppressor functions (384). Notch 1-4, are transmembrane receptors existing as a heterodimer pro-form. These interact with surface ligands such as delta, delta-like and jagged from another cell. Jagged-1 is overexpressed in metastatic prostate cancer (304, 398), and knockdown experiments revealed that its loss inhibits prostate cancer cell growth and forces S phase cell cycle arrest (398). In addition, constitutive expression of the active form of Notch 1 inhibits DU145, LNCaP and PC3 cell line proliferation (327).

Studies have shown that Notch signal transduction is important to normal prostate epithelial cell proliferation and differentiation (327, 377, 378). Also, prostate regrowth in castrated mice requires Notch 1 expression (378). A recent study showed that loss of
Notch signaling by using γ-secretase inhibitors, increased epithelial progenitor cell proliferation and impaired differentiation. These progenitor cells also expressed CK8 and CK14 markers of normal prostate stem and transit amplifying cells (295, 377). Thus, proper control of the Notch pathway appears to be important to regulate a balance between SC maintenance and activation of differentiation within the prostate.

1.6.4 Nanog, Oct3/4 and Sox2 transcriptional network

The transcription factors Nanog, Oct3/4 and Sox2 are important for self-renewal and inhibition of differentiation in embryonic stem (ES) cells (254). The expression of Oct3/4 and Nanog can be sustained by mechanisms such as Wnt pathway (306). Nanog, a homeobox transcription factor, blocks ES cell differentiation and thus regulates self renewal. The POU-domain protein Oct3/4 (octamer-binding transcription factor 34) can either activate or repress transcription depending on the promoter sequence context (271). It is a key regulator of pluripotency in mouse and human ES cells. Studies strongly suggest that Nanog/Oct3/4/Sox2 network is important in maintaining prostate CSCs (109, 141, 262). It was also recently reported that Nanog knockdown decreased the clonogenic growth and tumorigenicity of breast (MCF-7), colon (Colo320) and several prostate cancer (PC3, DU145 and LAPC-9) cell lines and xenografts (146, 147).

1.7 ONCOLYTIC VIROTHERAPY FOR PROSTATE CANCER

Development of therapies with non-conventional approaches is essential for treating CaP. Oncolytic virus therapy is an attractive means for treating prostate cancer. Replication competent viruses are used that can replicate, spread and exhibit oncolytic activity by a direct cytocidal effect (164). Oncolytic viruses are genetically engineered, naturally attenuated or non-pathogenic in humans so that they can replicate selectively in
cancer cells but do not harm normal tissues. The prostate gland is a nonessential organ and as such its complete removal or ablation is not life threatening (120). It is also easily accessible for inoculating viruses, obtaining tissue samples via a perineal or transrectal route. In addition, serum prostate-specific antigen (PSA) levels may be used to monitor the responses to therapy (120).

1.7.1 Adenovirus as oncolytic agent against prostate cancer

CN706 (also called CG7060 or CV706) was created by inserting the prostate specific enhancer (PSE), a minimal enhancer/promoter construct derived from the 5′ flank of the human PSA gene into the adenovirus type 5 (Ad5) genome so as to drive the E1A gene (297). When infected with CN706, human PSA-producing LNCaP prostate cancer cells expressed high levels of E1A but not non-PSA-producing DU145 prostate cancer cells. A single intratumoral injection with CN706 cured LNCaP xenografts and abolished PSA production in athymic mice. The initial dose-escalation phase I study was carried out in 20 patients with prostate cancer that recurred after radiotherapy (71). Another oncolytic adenovirus CV787 (also called CG7870) contains the rat probasin (207) promoter driven E1A gene and the PSE driven E1B gene together with a wild-type E3 region that suppresses the host immune system (394). CG7870 destroys PSA-producing cells 10000 times more efficiently than non-PSA-producing cells. A single tail vein injection with CG7870 eliminated LNCaP xenografts in athymic mice (73). This virus was administered intravenously as a single infusion in 23 patients with hormone refractory prostate cancer in a phase I trial (338). The combination of oncolytic virus therapy using CG7870 and radiation therapy was significantly more efficacious than either therapy alone (73).
Ryan et al. constructed an oncolytic adenovirus that contains the E2F-1 promoter driven E1A gene and the hTERT promoter driven E4 gene (OAS403) (301). The E2F-1 gene is a transcription factor that primarily upregulates genes associated with cell growth. OAS403 showed tumor selective cell killing in a panel of human cells. OAS403 was less toxic than a control virus that lacked the selective E4 control in human hepatocyte cultures as well as in animals. Systemic administration in mice with established LNCaP tumors resulted in a complete tumor regression at a tolerable dose in more than 80% of animals. Furthermore, the efficacy was significantly improved when the OAS403 therapy was combined with doxorubicin (301).

Ad-hOC-E1 is a conditionally replicating adenovirus whose replication is regulated by the human osteocalcin (hOC) promoter in order to target both epithelial and stromal cells of prostate cancer (149). Ad-Flk1-Fc is another virus that expresses a soluble vascular endothelial growth factor (VEGF) receptor capable of inhibiting angiogenesis and tumor growth (149). Ad-Flk1-Fc markedly inhibited the tubular formation of human umbilical vein endothelial cells (HUVEC) in vitro. In a subcutaneous C4-2 xenograft model, an 8-week treatment with either Ad-hOC-E1 or Ad-Flk1-Fc led to a 40–60% decrease in tumor volume compared with controls and furthermore the combination therapy resulted in a 90% clearance with three of ten animals showing complete tumor regression (149). Ad.D55.HRE has the deleted E1B55 gene in which the expression of E1A is regulated under the control of the hypoxia-response element (HRE) expression system (57). Ad.D55.HRE exhibited a significant anti tumor activity in athymic mice bearing PC-3 prostate cancer that expressed hypoxia-inducible factor (HIF)-1α (57).

1.7.2 Herpesviruses as oncolytic agents against prostate cancer
G207 was one of the first oncolytic HSV-1 strains taken into clinical trials. It was derived from HSV-1 strain F and has deletions in both copies of the g34.5 gene and a lacZ insertion inactivating the ICP6 gene. The double mutations permitted viral replication within cancer cells that can complement these mutations but not in normal cells including neurons (221). G207 has been shown to be effective against human prostate cancer in vitro and also in vivo following direct intraneoplastic inoculation (251) as well as intravenous administration (375). In athymic mice with human prostate cancer xenografts, intratumoral injection with G207 caused a reduction in tumor size and a complete eradication of more than 22% of tumors (375). Walker et al. reported that LNCaP tumors that recurred after radiation therapy remained sensitive to G207 therapy (375). In addition to G207, another oncolytic HSV-1 NV1020 was shown to cause cytolytic effects in all prostate cancer cell lines tested (60). Both viruses demonstrated a significant decrease in serum PSA and an inhibition of tumor growth when inoculated into PC-3 or C4-2 subcutaneous xenografts (60).

Fu et al. inserted the gene encoding a truncated form of the gibbon ape leukemia virus envelope fusogenic membrane glycoprotein (GALV.fus) into the genome of an oncolytic HSV-1 using an enforced ligation procedure (97). The expression of GALV.fus in the context of an oncolytic HSV-1 significantly enhanced the antitumor effect of the virus in DU145 prostate cancer cells. Furthermore, by controlling the GALV.fus expression by a strict late viral promoter, the glycoprotein was expressed in tumor cells but not in normal nondividing cells (97). In a lung metastasis model, intravenous administration of a doubly fusogenic oncolytic HSV (Synco-2D) significantly reduced the number of tumor nodules by day 40 (240). The oncolytic HSV-1 expressing murine
interleukin-12 (IL-12) (NV1042) also displayed greater efficacy than the control HSV-1 (NV1023) in two murine prostate cancer models (370).

1.7.3 Paramyxoviruses as oncolytic agents against prostate cancer

The most well characterized oncolytic strain of Newcastle disease virus is 73-T (2). A significant tumor growth inhibition (77–96%) was seen for prostate (PC-3), epidermoid (KB8-5-11), colon (SW620 and HT29), large cell lung (NCIH460), breast (SKBR3) and low passage colon (MM17387) carcinoma xenografts treated intratumorally with the strain 73-T (273). No patients with prostate cancer participated in the phase I clinical trials but phase II studies are ongoing for patients with cancer resistant to conventional modalities, including prostate cancer.

Respiratory syncytial virus (RSV) caused selective destruction of PC-3 cancer cells in vitro and in xenograft tumors in vivo due to apoptosis triggered by the downregulation of nuclear factor-kappaB (NF-kappaB) activity in infected cells (78). Kawaguchi et al. examined the direct tumor killing activity of inactivated Sendai virus particle (hemagglutinating virus of Japan envelope HVJ-E) in the hormone-resistant human prostate cancer cell lines PC-3 and DU145. HVJ-E infection produced interferon-α (INF-α) and INF-β and induced apoptosis in cancer cells. Direct injection of HVJ-E into PC3 tumor cells in SCID mice led to the reduction of the tumor volume and 85% of the mice were tumor free (155). A live attenuated strain of measles virus (MV) that infects cells exclusively through the PSMA receptor induced tumor regression of LNCaP and PC3 tumor xenografts (191). Vesicular stomatitis virus (VSV) is also a candidate oncolytic virus for prostate cancer. However, the efficacy of the virus depends on the type of infected cells. LNCaP cells were sensitive to infection with VSV, while PC-3 cells were
relatively resistant to VSV (46).

1.8 TARGETING CANCER STEM CELLS

Theoretically, the tumor therapy strategies focus on only a subpopulation of tumor cells rather than the bulk of the tumor. Conventional chemotherapy and radiation therapy seek to reduce the tumor burden, which is usually measured by immediate reduction in tumor size (123). With the understanding of the molecular events governing CSCs, it should be possible to develop therapeutics aimed at them. This is of paramount importance since the CSCs may mediate resistance and relapse of the most aggressive tumors to current treatments. This resistance may in part be the reactivation of several signaling cascades, such as sonic hedgehog, Wnt and Notch in the CSCs combined with increases in DNA repair mechanisms and ABC transporter-mediated multi-drug resistance (23, 25, 68, 70, 93, 101, 219, 300). In animal studies, labeled tumor cells (e.g., by GFP or luciferase) are transplanted and used to track metastasis and the outcomes of metastasis and various interventions (126). CXCR4 is one of the important molecules in both SCs and metastasis. Depletion of CD133+/CXCR4+ subpopulation using the anti-CXCR4 antibodies abrogated metastatic potential in a pancreatic cancer model (124). In another example, inhibition of the PTEN/Akt/PI3K signaling has been shown to reduce the CSC content and dampen the tumorigenic ability of CD133+/CD44+ prostate CSCs (77). These examples illustrate the clinical implications of CSC targeted therapeutic strategies, which when combined with conventional therapeutic regimens may deliver a long-lasting therapeutic efficacy and prevent tumor recurrence and distant metastasis.

1.8.1 Targeting ABC transporters

Chemotherapeutic agents are effluxed by mechanisms similar to those for Hoechst
33342 dye. This approach measures efflux of the Hoechst fluorescent dye, a substrate for ATP-binding cassette (ABC) transporters, out of the cells. Based on the observations that hematopoietic stem cells overexpress the ABC transporter ABCG2 (160), and that expression of this drug transporter inhibits differentiation of stem cells (399), it was hypothesized that the CSC should have higher expression of drug transporters, namely those responsible for multi-drug resistance. The isolation of cells based on their ability to efflux Hoechst 33342 was first observed using bone marrow aspirates by Goodell et al. in 1996. A small subpopulation of whole bone marrow cells were unstained and termed as the side population (SP). The SP was shown to exist in prostate epithelial cells isolated from men undergoing surgery for bladder outflow obstruction as a result of benign prostate disease (32). Further, the SP accounted for approximately 1% of prostate epithelial cells and was significantly reduced in the presence of verapamil, an inhibitor of ABC transporters.

Interestingly, there was no detectable SP in DU145, LNCaP, PC3 and PPC-1 cell lines (263). Currently, investigators agree that the SP represents an enrichment of CSCs but that it is not a pure population (113, 263). It is widely believed that this population contains not only the more primitive SC but also TA cells or cells further along the differentiation pathway. Blocking drug transporters in combination with administration of a chemotherapeutic agent may be effective at inducing cell death of CSCs simply by keeping the chemotherapeutic agent within the cell. A better understanding of exactly which transporters are highly expressed in prostate CSCs will aid in the selection of appropriate therapeutic agents.

1.8.2 Targeting the Sonic Hedgehog pathway
Cyclopamine targets smoothened, a G-protein receptor that mediates the Hh pathway. Treatment of mice bearing xenografted PC3 tumors with cyclopamine, resulted in apoptosis and tumor regression, and inhibited recurrence of the tumor for over 5 months (30). Furthermore, it was shown that treatment of PC3 CD44+ CSCs with cyclopamine results in decreased expression of MDR1 and ABCG2 suggesting that Hh signaling may also lead to a decrease in multi-drug resistance (331). Therefore, targeting Hh may kill CSC population and result in a greater toxicity to the cells by reducing multi-drug resistance.

1.8.3 Targeting the Niche of Stem cells

Calabrese et al., 2007 demonstrated that co-culturing of CSCs and endothelial cells resulted in proliferation of the CSCs that closely associated with the endothelial cells. In the case of normal prostatic epithelial SCs, it was shown that they reside proximal to the urethra at a band of smooth muscle cells that secrete TGFβ (364). The secretion of TGFβ in this area may be important in maintaining the niche for prostatic CSCs, as this factor is essential for promoting quiescence of SCs (302). Therefore, a greater understanding of not only the niche of prostate CSCs, but also of the factors and cells contributing to maintain this environment may help in devising better therapeutic strategies.

1.8.4 Targeting stem cell differentiation

If the prostatic CSCs could be pushed to differentiate into less quiescent, androgen responsive cells, then therapies efficacious against the entire tumor will be able to eradicate the CSCs. The patient can be cured and relapse prevented if the balance between self-renewal and differentiation pathways were tipped entirely towards differentiation. This would presumably lead to the loss of all CSCs. This concept has
been pondered by several investigators (180, 220) while, many questions regarding the pathways still remain to be answered.

The ability to study and understand the prostatic CSCs is hampered by several factors, including scarcity of the cells (1 %), a relative lack in culturing techniques that maintain the SC state and lack of adequate techniques that measure SC properties such as self-renewal. Traditional therapies for prostate cancer rely on both the removal of androgen as a stimulus and the ablation of rapidly cycling cells that have a limited ability to repair DNA. Given that the CSC is neither AR+ nor a fast-cycling cell, it is imperative that new therapies directed at the CSC need to be developed.

1.9 ONCOLYTIC VIROTHERAPY AGAINST CANCER STEM CELLS

Although the clinical responses observed with the current cancer therapies are encouraging, they are not entirely satisfactory (151, 202, 231, 232). The drawbacks could be due to unsuccessful targeting and elimination of the cells responsible for metastasis and recurrence. It is therefore, critical to fully characterize the nature and relationship between different populations of stem-like cells for efficient and long-term eradication of cancer. Tumor-initiating cells, also referred to as cancer stem cells, are thought to account for relapses following seemingly successful treatments because their slow turnover and capacity for expelling anti-tumor drugs leaves them untouched by conventional treatment regimens. Targeting of cancer stem cells might be the key for improving survival and producing cures in patients with metastatic tumors. Research should focus on developing tailored treatments by identifying patients who are likely to respond to a specific therapeutic regimen. Molecular and biochemical technologies including whole genome microarrays and mass spectrometry, respectively, could indicate the presence or absence
of tumor antigens aiding in informed decision to be made prior to therapy (235, 321).

Viruses enter cells through infection and might therefore not be sensitive to stem cell resistance mechanisms. During the last decades, oncolytic adenoviruses have been shown to effectively kill cancer cells, by seizing control of their DNA replication machinery and utilizing it for the production of new virions, ultimately resulting in the rupture of the cell. Few publications showed that adenovirus E1A mutant that targets the retinoblastoma-E2F transcriptional factor pathway (Delta-24) was able to kill CD133+ cancer stem cells or CD44 (+) / CD22 (low) cancer initiating cells in vitro and was also able to eradicate tumors derived from these cancer stem cells (148, 332, 334, 337). It was also reported that adenovirus serotype 3 was better than serotype 5 in infecting cancer stem cells in vitro (84, 148).

This ability to kill cancer stem cells does not seem to be limited to adenoviruses alone. A combination of low-dose etoposide with G47Δ (oncolytic herpes simplex virus) increased survival of mice-bearing intracranial human glioblastoma stem cell derived tumors without adverse side effects (56). While this is of interest, there are several issues that remain to be solved. As the population of cancer stem cells within a tumor is generally low (often less than 5%), it is a challenge for oncolytic viruses to ‘find’ and kill these cancer stem cells, especially when viruses are administered systemically. However, oncolytic viruses are the first approach shown to be effective against tumor-initiating cells and differentiated tumor cells. Virotherapy also sensitizes tumor cells to radiation and chemotherapy (150). Thus, oncolytic viruses hold significant promise for improving treatment options for patients with currently incurable cancer. The biggest obstacle in the field is the difficulty in translating approaches from the laboratory to patients and
unfortunately there are currently no clear solutions to this problem (122).
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Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 457:603-607.


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<th>Fusion protein cleavage site</th>
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<td>Not reported</td>
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Table 2. Naturally occurring NDV strains in human clinical trials

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<td>Infected tumor cell vaccine</td>
<td>(3, 4, 40, 183, 189, 250, 276, 316)</td>
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<td>MTH-68</td>
<td>Lytic/velogenic</td>
<td>Infectious virus</td>
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<td>Italien</td>
<td>Lytic/velogenic</td>
<td>Oncolysate vaccine/ infectious virus</td>
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<td>Hickman</td>
<td>Lytic/lentogenic</td>
<td>Infectious virus</td>
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<td>PV701</td>
<td>Lytic/lentogenic</td>
<td>Infectious virus</td>
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<td>HUJ</td>
<td>Lytic/lentogenic</td>
<td>Infectious virus</td>
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<td>La Sota</td>
<td>Lytic/lentogenic</td>
<td>Infectious tumor cell vaccine</td>
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Figure. 1. A schematic of Newcastle disease virus structure (image courtesy: Dr. Elankumaran Subbiah)
CHAPTER-2

Prostate Specific Antigen Retargeted Recombinant Newcastle Disease Virus for Prostate Cancer Virotherapy

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Abstract: Oncolytic virus (OV) therapies of cancer are based on the use of replication competent, tumor selective viruses with limited toxicity. Newcastle Disease Virus (NDV), an avian paramyxovirus, is a promising OV and is inherently tumor selective and cytotoxic only to tumor cells but replication restricted in normal cells. Despite encouraging Phase I/II clinical trials with NDV, further refinements for tumor specific targeting are needed to enhance its therapeutic index. Systemically delivered NDV fails to reach solid tumors in therapeutic concentration and also spreads poorly within the
tumors due to barriers including complement, innate immunity and extracellular matrix. Overcoming these hurdles is paramount to realize the exceptional oncolytic efficacy of NDV. We engineered the F protein of NDV and generated a recombinant NDV (rNDV) cleavable exclusively by prostate specific antigen (PSA). The rNDV replicated efficiently and specifically in prostate cancer (CaP) cells but failed to replicate in the absence of PSA. Further, PSA-cleavable rNDV caused specific lysis of androgen independent and dependent/responsive CaP cells with an EC$_{50}$ ranging from 0.01 to 0.1 multiplicity of infection. PSA retargeted NDV efficiently lysed three-dimensional prostaspheres, suggesting efficacy in vivo. Also, PSA-cleavable NDV failed to replicate in chicken embryos, indicating no pathogenicity to chickens. Prostate specific antigen targeting is likely to enhance the therapeutic index of rNDV owing to tumor restricted replication and enhanced fusogenicity.

**Keywords:** Oncolytic viruses-Newcastle disease virus-Prostate cancer-Prostate specific antigen-Retargeting.

**Introduction:**

Prostate cancer (CaP) is the second leading cause of cancer related deaths in the United States (25). Current treatment regimens for hormone resistant CaP are palliative and marginally increase survival. Oncolytic virotherapy is a novel approach for treating CaP and became particularly attractive with the advent of techniques for genetically manipulating RNA viruses (7, 21). Newcastle disease virus (NDV), an avian paramyxovirus, is an inherently tumor selective oncolytic virus (OV) with compelling
pre-clinical and phase I/II studies in human subjects (18,33,38). Although naturally occurring strains of NDV used as oncolytic agents have shown some promising results and have been used in clinical trials, genetic modification of NDV affords the opportunity to improve antitumor efficacy and its therapeutic index. A cleavage of the F protein is known to be required for initiation of infection and is considered to be a major determinant of NDV virulence (44).

We hypothesized that the fusion (F) protein cleavage site of NDV can be made cleavable by prostate specific antigen (PSA) to restrict its replication in PSA producing CaP cells. Therefore, we modified the F cleavage site to be activated and processed proteolytically by a prostate specific protease called prostate specific antigen (PSA). PSA is a serine protease with chymotrypsin-like substrate specificity (9, 37, 43) that is found in high concentrations (mg/ml) in the seminal plasma (36). Increased serum PSA levels correlate to tumor volume (49) and most probably result from leakage of PSA from the prostatic ductal system into the prostatic stroma and subsequently into the blood stream. The serum PSA test measures total PSA, which has been defined to consist of all immunodetectable PSA and comprises mostly of free PSA and PSA bound to the protein inhibitor α1-antichymotrypsin (ACT) (39). While some studies showed that the explant tissue from human prostate tumor had >80% of active PSA (14), others have shown that the prevalent form of PSA in tumor homogenates is the bound state (48). The therapeutic index of NDV can be enhanced by specific activation through cancer cell type specific proteases. To date, only oncolytic Sendai and measles viruses were replication restricted for tumor specific proteases such as matrix metalloproteases (MMP) or uroplasminogen (UPA) activator (28, 45).
NDV F protein cleavage site possesses the consensus amino acid sequence $^{112}$R/K-R-Q-R/K-R-F$^{117}$ for virulent and mesogenic strains and $^{112}$G/E-K/R-Q-G/E-R-L$^{117}$ for NDV strains of low virulence (9). To determine whether it would be possible to engineer rNDV targeted against tumor specific proteases, we performed F protein cleavage site modifications to that of PSA and examined the ability of the mutant rNDV to replicate and kill CaP cells specifically. PSA is serine protease with chymotrypsin-like substrate specificity (9, 1). Denmeade et al, (1997) reported that HSSKLQ as a PSA specific substrate which was not hydrolyzed by a variety of proteases including chymotrypsin and trypsin (13). Subsequently, they reported that Hp6$^{p6}$Sp5$^{p5}$Sp4$^{p4}$Kp3$Lp2$Qp1$//Lp1$’’ (// denotes the cleavage site) as the efficient substrate for PSA (34). Further, molecular docking studies indicated that the amino acids at P1-P3 are critical and any amino acid with a smaller side chain at P4 would allow substrate specificity (34). We therefore mutated the wild type fusion protein cleavage site sequence to $^{112}$RHSSKL$^{117}$, $^{112}$RRKLQL$^{117}$ and $^{112}$RRKLQF$^{117}$, to retain phenylalanine or leucine at the P1’ position because it was reported to be essential for the induction of fusion (41). We also constructed a F-Null protein in which the furin motif was replaced by the sequence $^{112}$GGPGGV$^{117}$ changing the P1’ amino acid to valine that would make it fusion defective (41) and by creating a neutral charge at the cleavage site with glycine. The peptide sequence of the NDV F-Null protein was therefore not expected to be cleaved by any known proteases and was used as a control protein in this study.

We were able to show that only $^{112}$RRKLQL$^{117}$ sequence at the cleavage site was PSA-specific and the recombinant NDV with PSA-specific site efficiently replicated and was cytotoxic to CaP cells and three-dimensional prostatsphere tumor mimics.
Materials and Methods:

Cells and reagents: Vero and androgen receptor negative (AR-) PC-3 and DU-145 prostate cancer (CaP) cell lines were purchased from ATCC and maintained in Dulbecco modified Eagle’s medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal calf serum (Invitrogen, USA). BSR-T7/5 cells were obtained from Dr. Griffith D. Parks, Wake Forest University and maintained in DMEM medium supplemented with 10% fetal calf serum and geneticin as described previously (6). Androgen receptor positive (AR+), PSA secreting C4-2b cells were originally obtained from Dr. Leland Chung, Emory University and were maintained in RPMI medium supplemented with 10% fetal calf serum. Another AR+, PSA secreting cell line WPE-int, was also obtained from ATCC and maintained in keratinocyte serum free medium (K-SFM) supplemented with 0.05mg/ml bovine pituitary extract (BPE) and 5ng/ml epidermal growth factor (EGF) (K-SFM media and supplements from Invitrogen, USA). AR+ and AR- CaP cells were supplemented with prostate specific antigen (Sigma Aldrich, USA) at 100ng/ml. A synthetic androgen analogue R1881 (Sigma Aldrich, USA) was used at 1nm/ml concentration, to induce intracellular PSA production only in androgen responsive AR+ cells. Cells were also maintained as non-adherent prostaspheres in serum free medium with 1x glutamine, 10ng/ml EGF and 1x B27 supplement without vitamin A (Invitrogen, USA). The full length Beaudette C antigenome and the nucleoprotein (NP), phosphoprotein (P) and large polymerase protein (L) support plasmids have been described elsewhere (16). Anti-NDV chicken serum and the goat-anti chicken HRP/FITC antibodies (Kirkegaard Perry Laboratories, USA) were used according to manufacturer’s instructions. Anti-F antibody, 39-D9 was a generous gift from Dr. Ronald Iorio,
University of Massachusetts School of Medicine. Tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin and tosyllysine chloromethyl ketone (TLCK) chymotrypsin were obtained from Sigma Aldrich, USA.

**Cell based assays:** The mutant F genes and the wild type (wt) HN were cloned into the Nhe I site of pCAGGS. Vero, BSR-T7/5 and PC-3 cells were seeded in 6-well plates and cotransfected at 80% confluence with 1μg of F and HN plasmids using Lipofectamine (Invitrogen). Prostate specific antigen (Sigma Aldrich, USA) was added to the wells transfected with F mutant plasmids, at a concentration of 100ng/ml, 12 hours post transfection. To determine the optimum PSA overlay concentration, PC-3 cells were cotransfected with mutant F and HN plasmids and supplemented with exogenous PSA (1 to 400 ng/ml) 12 hours post transfection. The optimum PSA overlay concentration was determined based on fusion index (11) and cell viability using trypan blue dye exclusion assay (3, 47). For determining the fusion index, PC-3 cells were cotransfected with wt or mutant F genes and the wt HN gene. At 72 hours post transfection, the cells were fixed using methanol: acetone (1:1) and washed with 1mM EDTA solution in phosphate buffered saline (PBS). After decanting PBS, cells were stained with hematoxylin-eosin stain (Fischer Scientific, USA). The fusion areas in eight different fields were counted to determine the fusion index (mean number of nuclei per cell) as described (24, 29).

**Generation and rescue of recombinant NDV:** Introduction of the KLQL, HSSKL, KLQF and Fnull cleavage site mutations, into the full length NDV BC genome was performed by a two round PCR directed mutagenesis. Primers were designed to introduce the desired mutations in the F cleavage site of a sub-clone carrying the Sac II to Not I segment of BC genome (31, 32). The sacII-notI fragment was then cloned into the full
length BC antigenome to generate BC-HSSKL, BC-KLQL, BC-KLQF and BC-Fnull full-length clones (16). Further, the SacII-PmII fragment bearing the EGFP gene (16) was cloned into BC-HSSKL, BC-KLQL, BC-KLQF and BC-Fnull full-length clones for obtaining the EGFP infectious clones. Transfection and rescue of recombinant NDVs were performed as described previously (31, 32) with minor modifications. Briefly, cells were transfected with full-length infectious clones of NDV along with NP, P and L plasmids, with or without wild type BC F gene. An exogenous overlay of 100ng/ml prostate specific antigen (PSA) 48 hours prior to and after transfection of BSR-T7/5 cells was made. BSR-T7/5 cells were overlaid with fresh Vero cells 4 days post transfection and the cells were examined for syncytia and fluorescence. The entire cell lysate, 7 days post transfection was used for amplifying the virus in DU145 cells.

**Immunofluorescence assay (IFA) and flow cytometry:** Vero cells were transfected with wt F or mutant F genes, with or without the HN gene. Following 48 hours post transfection, cells were fixed with methanol: acetone (1:1) for 30 minutes at room temperature. Live cell staining was carried out for cells transfected with wt or mutant F proteins to detect cell surface fluorescence. Cells were washed with PBS and blocked in PBS containing 1% BSA (Sigma Aldrich, USA) and 0.02% sodium azide (Sigma Aldrich, USA) for 30 minutes on ice. Blocking was followed by incubation with primary antibody 3-9D9 (diluted 1:500 in PBS containing 1%BSA and 0.02% sodium azide, FACS buffer) for 1 hour at 4°C. After three washes with FACS buffer, cells were incubated for 1 hour at 4°C with goat anti-chicken-FITC secondary antibody (Kirkegaard Perry Laboratories, USA) (1:100 diluted in FACS buffer). Following three more washes with FACS buffer, fixed cells were observed under a
fluorescence microscope while live cells were resuspended in FACS buffer and subjected to flow cytometry.

**Immunoblotting:** Virus infected or plasmid transfected cell lysates were subjected to 10% SDS-PAGE under non-reducing conditions. Following electrophoretic separation, the gels were subsequently transferred to nylon membranes using the iblot kit (Invitrogen, USA) as per manufacturer’s instructions. The membranes were then blocked in PBS containing 0.5% Tween 20 and 1% nonfat dried milk for 2-3 hours at room temperature or overnight at 4°C. Membranes were washed in PBS-Tween 20 and incubated with primary antibody, anti-NDV chicken serum (purchased from Kirkegaard Perry Laboratories, USA) diluted in PBS-Tween 20 and 0.5% nonfat milk (1:2000) overnight at 4°C. Membranes were further washed and incubated in secondary goat anti-chicken IgG coupled to horseradish peroxidase (Kirkegaard Perry Laboratories, USA) diluted in PBS-Tween and 0.5% nonfat milk (1:20,000), for 1 hour at room temperature. Membranes were washed extensively and bound antibody was detected using the TMB peroxidase substrate (Kirkegaard Perry Laboratories, USA).

**Virus titration:** Supernatants from virus-infected DU145 cells were clarified at 3,000 xg and used as virus stock. Virus titers were obtained by calculating the TCID$_{50}$ using Reed and Muench method as described (17).

**Mean death time:** Ten 10-day-old specific pathogen free chicken embryos were each inoculated with five different dilutions of the test viruses for calculating the mean death time as described (2).

**Growth kinetics:** Cells and spheres were seeded in 6-well plates at 5x10$^5$ cells per well and infected with recombinant BC-KLQL-GFP at a multiplicity of infection (MOI) of
0.01, 0.1, 1 and 10 MOI for multicycle growth studies. After virus adsorption for 1 hour at 37 °C, cells were washed with PBS to remove any unabsorbed virus and serum free medium containing PSA (100ng/ml) or R1881 (1nm/ml) was added. At various time points after infection, 100 μl of supernatants were removed and the TCID$_{50}$ were determined by infecting fresh DU145 cells.

**Reverse transcription PCR and sequencing:** Undiluted BC-KLQL-GFP virus was serially propagated ten times in WPE-int cells. To analyze the stability of the introduced F mutation, F-KLQL sequence was confirmed by performing RT-PCR of infectious supernatant using NDV genome specific primers spanning the M-F region.

**Cell viability:** Cells and spheres were plated as five replicates in 6-well plates at 5x10$^5$ cells per well and infected with recombinant BC-KLQL-GFP at 0.01, 0.1, 1.0 and 10.0 MOI. Cells and spheres were trypsinized at 24, 48, 72, 96 and 120 hours post infection and checked for viability using the trypan blue dye exclusion assay (3, 47).

**Statistical analysis:** One-way analysis of variance and the student t tests were performed using the JMP 9 software. EC$_{50}$ values were calculated using the dose response versus inhibitor analysis with four parameters, Graphpad prism 5 software.

**Results and Discussion:**

We had shown that recombinant Beaudette C (rBC) strain of Newcastle disease virus (NDV) specifically kills human tumor cells while sparing normal cells in an interferon independent manner. We found that NDV kills tumor cells by intrinsic and extrinsic pathways of apoptosis (16). We had also shown that rBC is safe and inherently oncolytic in a preclinical mouse model. We demonstrated that a single dose of interferon resistant and sensitive recombinant NDV effectively eradicated tumor burden in human
fibrosarcoma xenografts in nude mice (15). Recently, we reported that the absence of retinoic acid-inducible gene I (RIG-I), a cytosolic RNA sensor, determined sensitivity to NDV and proinflammatory cytokines and chemokines were altered differentially in infected normal and tumor cells (5).

**NDV fusion protein mutants are transported to the cell surface.** The F cleavage site mutants HSSKL, KLQL, KLQF and Fnull were successfully cloned into the multiple cloning site of expression vector pCAGGS and confirmed for correctness by sequencing (Fig. 1A). We screened all three NDV fusion protein mutants with putative PSA cleavable motifs and a null mutant, in a plasmid system for their PSA specificity. Immunofluorescence assay was performed to analyze the fusion protein mutants for cell surface expression, and the percentage fluorescence was quantified by flow cytometry. All fusion proteins, except the KLQF mutant, were transported to the cell surface. Percentage fluorescence was calculated by normalizing all the values for mock-transfected cells (Fig. 1B). Co-expression of wild type (wt) NDV F and hemagglutinin (HN) proteins in Vero cells resulted in multinucleated syncytia. The mutant proteins, however, were not fusogenic (Fig. 1C). This suggested that the mutant fusion proteins were functionally inactive and not cleaved by ubiquitous proteases. The F protein of virulent NDV strains with multibasic cleavage site amino acids are cleaved by ubiquitous subtilisin-like proteases such as furin, PC6 and PACE 4, while non-pathogenic strains with monobasic cleavage site are cleaved by trypsin-like enzymes found only in selected tissues (10, 12). Modification of the NDV F cleavage site to HSSKL and KLQL did not interfere with protein synthesis, maturation or cell surface transport pathways.
**KLQL mutant is activated by exogenous PSA overlay.** To further analyze the functional properties of mutant fusion proteins, cell culture medium was supplemented with PSA at 12, 24 and 48 hours post-transfection. The addition of PSA (100 ng/ml) resulted in efficient cleavage of the KLQL but not HSSKL mutant (Fig. 1D). It was found that exogenous supplementation of PSA at a concentration 100 ng/ml was well tolerated by the cells and a mean fusion index of 17.78, similar to the wild type F plasmid (data not shown) was observed with the KLQL mutant (Fig. 1E). Fusion index did not increase when PSA concentrations more than 100ng/ml were used. At higher doses, PSA was cytotoxic to cells, which could be due to the chymotrypsin-like protease activity of PSA on adherent cells. The HSSKL mutant F protein was defective in forming syncytia even after a PSA overlay, while the KLQL mutant was fusogenic (Fig. 1F). This indicated that the KLQL mutant F protein was activated by PSA and can be considered an ideal PSA substrate.

**Recombinant NDV is replication restricted for PSA.** Rescue of recombinant NDV was carried out using available reverse genetics tools as previously described (31). The modification to the protocol included an exogenous overlay of PSA (100ng/ml) 48 hrs prior to and after transfection. Recombinant NDV (rNDV) with HSSKL motif could be recovered only by pseudotyping with NDV wt F and failed to undergo multicycle replication in CaP cells (Fig. 2A). The rNDV with KLQL motif (BC-KLQL-GFP) was successfully recovered by exogenous PSA supplementation in BSR-T7/5 cells (Fig. 2A). Also, BC-KLQL-GFP replicated only when PSA was added to the medium of Vero and WPE-int cells (Fig. 2B).

To confirm whether virus replication was dependent on active PSA, we inactivated
PSA with 10% fetal bovine serum (FBS). Human and animal sera are enriched with protease inhibitors including serpins (serine protease inhibitors) that bind and inactivate proteases including trypsin and chymotrypsin (20, 42). It is well known that the proteolytic activity of PSA is inhibited in the bloodstream by the formation of complexes with serine protease inhibitors such as alpha-1 antichymotrypsin and others. It was found that the multicycle virus replication of BC-KLQL-GFP was severely impaired with the addition of FBS (Fig. 2, B and C), indicating PSA-specificity. Exogenous TPCK trypsin (0.5 μg/mL) or TLCK chymotrypsin (1 μg/mL) overlay did not support virus replication as well (data not shown). This further confirmed PSA specificity of the recombinant BC-KLQL-GFP virus.

**PSA cleavage motif was stable and the PSA mutant virus was non-pathogenic and failed to replicate in chicken embryos.** The PSA cleavable KLQL mutation was stable even after ten serial passages of BC-KLQL-GFP in androgen receptor positive WPE-int cells (Fig. 2D). The serial passages were carried out in an androgen responsive cell line because intracellular PSA production could be induced using R1881 (synthetic androgen analog). The mean death time following BC-KLQL-GFP infection in 10-day-old specific pathogen free (SPF) chicken embryos was greater than 168 hrs and no embryo death could be recorded. Viral RNA was not detected in the allantoic fluid of infected SPF embryos, indicating absence of virus replication (Fig. 2E). This shows that BC-KLQL-GFP does not replicate in SPF chicken embryos and is non-infectious to chickens.

**PSA targeted recombinant NDV replicates in prostaspheres.** To determine whether PSA mutant would replicate in *in vitro* mimics of tumors, we performed growth kinetics in CaP cells and prostaspheres. CaP cells were cultured in serum free media to form
Prostaspheres represent 3D clusters of tumor cells that develop into multicellular aggregates of fairly large size. Spheroids contain different subpopulations of cells that can be quiescent, hypoxic, and necrotic closely mimicking a tumor (19, 30, 46). Spheres were shown to be promising in vitro models for testing various anti-cancer compounds, as they represent better cellular dynamics and architecture than two-dimensional (2D) monolayer cultures (22, 23, 26, 35). PSA mutant virus replication was dependent on the multiplicity of infection (MOI) in androgen receptor positive (AR+) DU-145, PC-3 and negative (AR-) C4-2B and WPE-int CaP cells or spheres. As expected, virus replication increased with MOI in all the cell types (data not shown).

The androgen independent cell line DU-145 was the most permissive cell type (Fig. 3A) and the virus yield using exogenous PSA overlay in AR+ and AR- cells and most spheres were significantly different between 1 and 10 MOI (p<0.0001). Interestingly, induction of intracellular PSA in androgen dependent AR+ cells (WPE-int and C4-2B) resulted in significant enhanced titers (Fig. 3B). Engaging the AR with a synthetic androgen analog R1881 induced intracellular PSA secretion (4) and thereby increased virus replication (1 MOI) in AR+, WPE-int and C4-2B cells (Fig. 3C), to levels comparable with 10 MOI using exogenous PSA (data not shown). On the other hand, AR+ prostaspheres disaggregated in the presence of R1881 leading to low virus yields and non-specific cell death (Fig. 3C, 4). The remarkable fusogenicity in WPE-int and C4-2B androgen responding cells suggested an ability to spread efficiently and overcome intratumoral interstitial pressure (Fig. 4).

**PSA mutant was cytotoxic to CaP cells and prostaspheres.** Trypan blue cell viability assays
were performed for virus-infected CaP cells and spheres. The EC$_{50}$ ranged from 0.01 MOI for AR+ cells/spheres to 0.1 MOI for AR- cells/spheres at 120 hours post infection (Fig. 5, A and B). Cytotoxicity was significantly enhanced by R1881 induction in WPE-int AR+ cells and marginally enhanced in C4-2B AR+ cells (Fig. 5C). Differential tumor selectivity may be due to the variations in cellular antiviral response to infection with NDV and RIG-I expression (5). These results demonstrate that the PSA cleavable rNDV will be activated only in the CaP tumor microenvironment, undergo selective multicycle replication and mediate cytotoxicity only in PSA producing cells and intracellular F cleavage is important for viral oncolysis.

CaP is especially suited as a target for OV$s$ because the prostate gland is easily accessible for inoculating viruses or for obtaining tissue samples. PSA, a serine protease, is abundant in the seminal plasma and increased serum PSA levels are known to correlate to prostate tumor volume (9, 36, 37, 43, 49). A major setback in using naturally occurring OV$s$ for cancer therapy is off targeting and failure to reach maximum tolerated doses. As most of the proteolytically active PSA is present only in CaP (1, 9, 37), off targeting will result in an abortive replication cycle with PSA dependent rNDV. Although evidence exists to support PSA targeted prodrugs (8, 13, 27), we have obtained “proof of principle” for a concept that a recombinant virus could be replication restricted for PSA. Tumor-specific targeting of rNDV can be combined with its inherent oncolytic and immunostimulatory properties, resulting in enhanced therapeutic index by tumor specific replication and efficient intratumoral spread. During the course of our study, the mesogenic Beaudette C strain was classified as a “Select agent” by the United States Department of Agriculture, with delayed regulatory approvals for performing preclinical
tumor regression studies with rNDV in mouse models. Select Agents are federally regulated agents that have potential use in biological warfare. Although we were restricted by select agent regulations, our results of virus replication and cytotoxicity in a range of AR+ and AR- CaP cells and 3-D prostaspheres suggest that PSA cleavable rNDV is a promising candidate for immediate preclinical and phase I clinical trials.

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References:


Fig. 1. Characterization of fusion protein mutants using cell based assays. (A) A schematic of the Newcastle disease virus (NDV) fusion protein and the PSA activation mutants. (B) Cell surface expression was measured quantitatively by flow cytometry. Note HSSKL, KLQL and Fnull proteins were transported to the cell surface, while KLQF was undetectable. (C) Immunofluorescence staining of Vero cells cotransfected with NDV F and HN plasmids, using antibody against F protein (10x magnification). Syncytia observed only with wild type F) (D) Activation of of mutant fusion proteins with exogenous PSA overlay (100ng/ml) compared to activation of wild type fusion protein with no overlay. Lysates collected at 24 and 48 hours post transfection were used to detect the F1 peptide using anti-NDV polyclonal chicken serum. The KLQL mutant was cleaved by PSA and PSA activation was as efficient as the wild type fusion protein activation by ubiquitous cell proteases (E) Determination of optimal PSA overlay concentration. Exogenous PSA overlay concentration was calculated based on the percentage cell viability and fusion index in PC-3 cells. Co-transfection of expression plasmids (KLQL fusion mutant and hemagglutinin) in PC-3 cells, followed by an exogenous PSA overlay (1ng/ml to 400ng/ml) resulted in variable fusion index and cell viability. Results are represented as mean ± standard deviation of five independent experiments. (F) Syncytia formation in BHK-T7 cells 72 hours post-transfection. Note the defective fusogenicity for HSSKL mutant.
Fig. 2. Specificity of recombinant NDV to PSA. (A) Virus rescue and sequential passage of BC-HSSKL-GFP and BC-KLQL-GFP mutant viruses in BHK-T7 cells (20x magnification). Note the absence of multicycle replication in HSSKL virus. (B and C) Replication kinetics of BC-KLQL-GFP virus (MOI 1) in Vero and WPE-int cells with or without PSA overlay, with PSA + 10% FBS supplementation. (D) Stability of KLQL mutation after ten serial passages (MOI 1) in WPE-int cells. (E) Reverse transcription PCR gel confirming absence of viral RNA in the allantoic fluid of infected specific pathogen free chicken embryos, Lanes a) DNA marker, b) allantoic fluid from embryo infected with Lasota-EGFP, c) Cell culture supernatant infected with BC-KLQL-GFP, d and e) allantoic fluid from embryos infected with BC-KLQL-GFP.
Fig. 3. Virus yield in prostate cancer cells and spheres. A) Virus yield in DU145 cells was significantly higher than in other cells. Spheres are designated as DU145S, PC-3S, WPE-intS and C4-2BS. B) PSA induction using synthetic androgen analog R1881, significantly increased virus replication in AR+ cells (C4-2B and WPE-int) and decreased virus yield in AR+ spheres (C4-2BS and WPE-intS), * indicates P< 0.0001, n=8. C) Multistep growth kinetics of BC-KLQL-GFP, MOI of 1, after induction of PSA using R1881 (1nm/ml).
Fig. 4. BC-KLQL-GFP infection of AR+ and AR- cells and spheres, MOI 1, 72 hours post-infection.
Fig. 5. Cytotoxicity of PSA-cleavable NDV in prostate cancer cells and spheres. Cell viability was assessed by the trypan blue dye exclusion assay at 120 hours post infection and EC$_{50}$ ≥ MOI, 120 hours post infection, was calculated by the sigmoidal dose response versus inhibitor analysis with four parameters, using the GraphPad Prism 5 (GraphPad Software). A) AR- cell and spheres, B) AR+ cells and spheres. C) Percentage cell viability with R1881 was significantly lower than PSA overlay, ** indicates P<0.0002, * indicates P<0.0001, n=6.
CHAPTER- 3

Oncolytic Virotherapy Using a Prostate Specific Antigen Activated Recombinant Newcastle Disease Virus Against Prostate Tumor Initiating Cells

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Abstract:

Background: Currently, androgen depletion is an essential strategy for treating human prostate cancer. Despite the progress in cancer stem cell research, hormone independent stem cells escaping conventional treatment presents a major therapeutic challenge. Oncolytic virotherapy is a promising novel approach that overcomes the limitations posed by radiation and chemotherapy. In this study, the oncolytic efficacy of a recombinant paramyxovirus against prostate cancer stem-like/tumor initiating cells was evaluated.
**Methods:** An androgen independent DU145 prostate cancer cell line was analyzed for prostasphere generation, proliferation and self-renewal potential *in vitro*. The tumor initiating ability of prostaspheres was tested *in vivo* by subcutaneous xenografts in nude mice. The cancer stem cell marker profile of cell line derived spheres and xenografted tumor derived spheres were compared by confocal microscopy. Replication kinetics and cytotoxicity of recombinant Newcastle disease virus in primary tumor derived prostaspheres was assessed by multistep growth curve and cell viability assays, respectively.

**Results:** Prostaspheres generated from androgen receptor negative DU-145 xenograft tumors showed self-renewal and clonogenic potential *in vitro*. Embryonic stem and progenitor cell markers like nanog, nestin and CD44 were overexpressed in prostaspheres in serum-free medium as compared to the DU 145 cell line in serum containing medium. Prostaspheres exhibited increased tumorigenicity *in vivo* after primary or secondary xenografts, suggesting that they contain putative tumor initiating cells. Serial xenotransplanted tumor and cell line derived prostaspheres were permissive for recombinant Newcastle disease virus replication, when the fusion protein was activated by exogenous prostate specific antigen. An effective concentration (EC$_{50}$) of 0.11-0.14 multiplicity of infection was sufficient to cause 50% prostasphere cell death in cell culture.

**Conclusion:** Oncolytic virotherapy using recombinant Newcastle disease virus is a promising approach to target prostate tumor initiating cells.

**Key words:** Prostasphere; cancer stem cell; Newcastle disease virus; oncolytic virotherapy.
**Introduction:**

Prostate cancer (CaP) is the most common cancer affecting man and the second leading cause of cancer-related deaths in males in the developed world (18, 53). The disease progresses from intra-epithelial neoplasia to hormone refractory prostate cancer (HRPC) and HRPCs contribute to CaP associated deaths (25, 34). It was hypothesized that the adult prostate contains stem, transit amplifying and post-mitotic cells and that stem cells were androgen independent for survival (17). Prostate epithelium is traditionally considered to be composed of three major cell types, luminal, basal and neuroendocrine cells based on specific patterns of cytokeratin expression. Prostatic epithelial cells are also known to coexpress both basal and luminal cell-associated markers (16, 46-48, 51). Although the mechanisms responsible for CaP remain elusive, prostate cancer stem cells (PSCs) are widely regarded to be the origin of CaP initiation and progression (20, 22, 42). Multiple cell types in the prostate gland have been considered to be potential cells of origin for tumor development (21, 23, 44, 50).

Cancer stem cells (CSCs) share surface antigens with their tissue stem cell counterparts (1, 27, 33, 40, 41). A small population of primary CaP cells expressing the surface antigenic profile CD44+ $\alpha_2\beta_1^{hi}$CD133+ was suggested to be a candidate for CaP SCs (6). The CD44+ subpopulations isolated from several CaP cell lines were tumorigenic in nude mice than the isogenic CD44- population (29). However, it has to be demonstrated if CD44+ $\alpha_2\beta_1^{hi}$CD133+ cells isolated from primary CaP tissues are capable of initiating tumors in immunocompromised mice. Another report suggested that an undifferentiated subtype of basal cells, based on the co-expression of the human pluripotent stem cell marker TRA-1-60 with CD151 and CD166, recapitulated the original parent tumor in
serial xenotransplantations (32). The challenge now is to relate marker expression with stem cells and cell lineages.

CaP SCs are thought to be responsible for tumorigenesis, tumor maintenance and recurrence due to inherent resistance to current treatment modalities such as chemotherapy and radiation. A small number of CaP SCs can thrive after anti-androgen treatment and become lethal even with castrate levels of testosterone (24, 36). CSCs bare many similarities with normal stem cells such as self-renewal, multipotency, relative quiescence and cytoprotective mechanisms like activation of DNA repair mechanisms and expression of drug transporters (8). Presence of these cytoprotective mechanisms renders immunity to CSCs from cytotoxic therapy, making them very attractive targets for future cancer therapy. Oncolytic virotherapy (OV) offers a novel approach for eradicating CSCs using mechanisms of cell killing that differ from conventional therapies. In virotherapy, oncolysis is achieved by cell lysis through intracellular virus replication. Also, anti-tumor effect of oncolytic viruses is hypothesized to be a fine interplay between anti-tumor and anti-viral immune responses (12, 28).

In the present study, cells and spheres derived from androgen receptor negative (AR-), DU-145 cell line and its primary xenograft tumors were analyzed for a putative cancer stem cell population by sphere formation, self-renewal, proliferation and clonogenicity assays. We show that prostaspheres were enriched in cancer stem-like cells compared to cell monolayers, as evidenced by their stem cell marker profile, self-renewal, clonogenicity and in vivo tumorigenic potential. Further, when cultured in the presence of prostate specific antigen (PSA), spheroids supported the replication of a PSA activated
recombinant Newcastle disease virus (rNDV). Virus replication resulted in selective
cytotoxicity with an EC$_{50}$ of ~ 0.1 multiplicity of infection (MOI).

**Materials and Methods:**

**Prostate Cancer Cell Lines and Primary Xenograft Tumor Derived Cells**

Androgen receptor negative (AR-) DU-145 CaP cell line was purchased from America
type culture collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium
(DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS). DU-145 xenograft
tumors were harvested, minced with disposable scalpels in RPMI and digested with 0.5
mg/ml collagenase type I A (Sigma) and 100 U/ml RNase-free DNase (Promega) to
reduce cell clumping during dissociation for 90 min at 37°C with constant agitation.
Dissociated tissue was passed through 100 µm cell strainers (BD Falcon). The resulting
cells were pelleted at 800 xg and live cell numbers were determined as a function of
trypan blue exclusion (32). The cells were plated and passaged to generate prostaspheres.

**In Vitro Prostasphere Culture**

Monolayer and primary tumor cells were digested using Accutase (Sigma) and
resuspended in serum-free neurobasal medium (Invitrogen) containing 1x glutamax
(Invitrogen), 1x B27 lacking vitamin A (Invitrogen) and supplemented with 10 ng/ml
recombinant epidermal growth factor (EGF), (Invitrogen). Typical spheres formed in 3
days. Spheres were subcultured using Accutase, counted and resuspended in the above
media. To assay the proliferative capacity of secondary and tertiary spheres, cells were
dissociated using Accutase, plated at a clonal density of 2x10$^5$ cells/5ml in 25 cm$^2$ flasks
and counted after 7 days.
Soft Agar Clonogenicity Assay

DU-145 monolayer and xenografted tumor derived cells were plated in individual wells of a six-well plate at densities $4 \times 10^2$, $4 \times 10^3$ and $4 \times 10^4$ cells/well in media containing 0.3% agarose as described (52). Wells were stained with neutral red after 21 days and colonies were counted under a phase contrast microscope. Triplicates were used for each density and the mean number of colonies formed was calculated from three independent experiments.

Cancer Stem Cell Markers

Immunofluorescence staining of monolayer or primary tumor cells and prostaspheres was performed by fixing cells with methanol:acetone (1:1) solution for 10 min at -20°C, followed by staining with the specific primary antibodies. Spheres were allowed to settle in a 10% FCS containing medium (DMEM) overnight at 37°C, before fixing. The primary antibodies used include anti-human CD133/1 (AC133, Miltenyi Biotec, 130-080-801), anti-human CD44 (BD Biosciences, 561858), anti-human TRA-I-60 (Biovision, 3079-25), anti-CK14 and anti-CK18 mouse monoclonal antibodies (Santa Cruz, sc-53253 and sc-51582, respectively), rabbit anti-Nanog (Santa Cruz, sc-33760) and anti-Sox2 (Santa Cruz, sc-17320) antibodies, mouse monoclonal anti-human ABCG2 (Stem cell technologies, 01428) and anti-Nestin mouse monoclonal antibody (BD Biosciences, 561230). Slides were incubated in the dark with primary antibodies for 1 hour at 37°C. Chamber slides were subsequently washed in 1x phosphate buffered saline tween-20 (PBST) and incubated with secondary antibodies for 1 hour at 37°C. Slides were then washed and mounted with a coverslip using Vectashield mounting medium with 4′6-Diaminido-2-phenylindole (DAPI) (Vector laboratories). Images were captured using a
laser-scanning microscope (Carl Zeiss LSM 510). DU-145 monolayer cells were dissociated using Accutase and pelleted by brief centrifugation at 1000 rpm. Cells were then stained with anti-human ABCG2 (Stem cell technologies, 01428) antibody for 1 hr at 4°C, washed twice with PBS and sorted by fluorescence activated cell sorting.

**Tumor xenotransplantation**

Female athymic nude mice, strain Foxn1nu (Harlan Laboratories), aged 4-5 weeks were transplanted with 1x 10^2, 1x10^4 and 1x10^6 prostaticspheres or monolayer cells in 100 μl of phosphate buffered saline as subcutaneous tumors. All tumor induction ad subsequent monitoring were according to approved VirginiaTech IACUC tumor protocols. Mice were inspected for tumors and the tumor size was measured every two days using a vernier caliper. The tumor volume was determined using the formula L*W^2/2, where L and W are the longest and the shortest diameters of the tumor mass, respectively. Tumors were harvested and the primary tumor derived cells and spheres were subcutaneously transplanted into a new set of nude mice to produce secondary xenografts.

**Multi-step Growth Kinetics of rNDV**

Prostaticspheres were seeded in 6-well plates at 5x10^5 cells per well and infected with rNDV (BC-KLQL-GFP) at a multiplicity of infection (MOI) of 0.01, 0.1, 1 and for multi-step growth kinetics. After virus adsorption for 1 hr at 37°C, cells were supplemented with serum free medium containing PSA (100 ng/ml). At designated time points after infection, 100 μl of infected cell supernatants were removed and the mean tissue culture infective dose (TCID_{50}) was determined on DU145 cells. Virus titers were obtained by calculating the TCID_{50} using Reed and Muench method as described (11).

**Cytotoxicity by Trypan Blue Dye Exclusion Assay**
Prostaspheres were plated as five replicates in 6-well plates at a density of $5 \times 10^5$ cells/well and infected with rNDV at 0.01, 0.1, 1.0 and 10.0 MOI. Cells were trypsinized at 24, 48, 72, 96 and 120 hr post infection and cell viability was determined using the trypan blue dye exclusion assay (2, 43).

**Statistical Analysis**

Statistical analyses were performed using JMP 9 for Mac software. Data were presented as mean ± standard deviation unless otherwise stated. All Student’s t-tests performed were two-tailed and a p-value < 0.05 was considered statistically significant. Power analysis was also carried out using JMP 9 to determine the accuracy of replicates for a given experiment. The 50% effective concentration (EC50) values were calculated using the dose versus inhibitor response curve with four parameters using Graphpad prism software (Graph Pad Inc.).

**Results:**

**Prostaspheres derived from DU-145 primary xenograft tumors self-renew, proliferate and are clonogenic.**

To investigate whether sphere-forming cells may be obtained from AR- cell line, DU-145, monolayer cells were dissociated to form a single cell suspension and cultured in serum free neurobasal medium with defined supplements. Typical prostaspheres formed in 3 days, which increased in size gradually and appeared like fused spheroids within 7-10 days (Fig. 1A). Although, primary spheres could be generated from DU-145 cell line, secondary and tertiary spheres could be generated only from DU-145 tumor xenografts (Fig. 1B). The proliferation rates of spheres derived from ABCG2- population induced xenografts and sphere xenografts were significantly different (p<0.0001) (Fig. 1B).
To determine anchorage independent growth and clonogenicity, soft agar assay was performed with DU-145 cell line and xenograft derived cells. Prostaspheres from monolayer cells and xenografts were found to be morphologically similar and formed small colonies in soft agar (Fig. 1C). Interestingly, DU-145 xenograft derived cells formed colonies with a clonal density of as low as 400 cells/well. However, power analysis revealed that a clonal density of 1000 cells/well was most accurate to compare cells seeded per colony formed, statistically, between the cell line and tumor derived cells (power = 0.6562). DU-145 cell line was slightly more clonogenic than DU-145 sphere xenograft derived cells (p <0.0157) (Fig. 1D), suggesting that cells grown in serum containing medium tend to be clonal.

These *in vitro* studies indicated that the xenograft cells could grow in an anchorage independent fashion and the spheres generated were enriched for a population of self-renewing cells that proliferate.

Spheres Exhibit Tumorigenicity in Immunodeficient Mice

To evaluate whether prostaspheres derived from DU145 cell line can induce tumors, 1x10^2, 1x10^4 and 1x10^6 cells/100 µl were subcutaneously implanted in athymic nude mice. Table I shows the number of mice that developed palpable tumors and the latency associated for tumor development. One out of ten mice injected with 1x10^4 DU-145 prostaspheres developed a tumor in 37 days, and one out of five mice injected with 1x10^6 cells had a tumor in 30 days. On the other hand, no tumors could be detected in the groups that received DU-145 monolayer cells even after 60 days post injection. This indicates that prostaspheres are more tumorigenic *in vivo* as compared to the heterogeneous cell population in the DU-145 cell line. Interestingly, two out of ten mice
that received $1 \times 10^4$ DU-145 ABCG2- cells formed tumors at 35 and 50 days respectively. This result was consistent with another report suggesting that the ABCG2- population contains primitive stem-like cancer cells (30). An increase in the latency period was found to enhance tumor progression in ABCG2- group.

To assess the ability to form secondary tumors, sphere-derived xenografts were harvested and grown as cells or spheres for one passage. Cells and spheres from xenografted spheres were implanted subcutaneously into athymic female nude mice for secondary xenotransplants (Table 1). All mice in the sphere xenograft derived cells/spheres groups developed tumors irrespective of whether they were grown as cells in serum containing medium or as spheres in serum free medium. Once again, a longer latency period was associated with increased tumor proliferation. This indicated that self renewing stem like cells are quiescent, slow cycling cells.

**Prostaspheres Predominately Express Stem Cell Markers**

The prostate epithelium is composed of basal cells, stem cells and transit amplifying cells, luminal cells and neuroendocrine cells (7). Prostate epithelial cells can be differentiated based on the expression of cytokeratin and other markers (45). As prostaspheres were significantly tumorigenic than their parent cell line, we sought to determine the marker expression profile of prostaspheres. Immunostaining was performed using antibodies against known cytokeratin and stem cell markers. DU-145 monolayer cells and primary tumor derived cells uniformly expressed CK18, a luminal cell differentiation marker. Expression of the basal cell marker CK14 was less intense. Spheres on the contrary, were negative for CK14 expression except for a few cells on the periphery of the spheroids (Fig. 2) but expressed CK18 to similar levels as that of cells.
This led us to perform a Z-stack analysis of spheroids stained for CK14 cytokeratin. It was observed that prostasphere cells in the center of the spheroid showed reduced or no expression of the basal differentiation marker CK14 (Fig. 3A).

Western blot analysis showed an increased expression of the transcription factor Nanog, in xenograft spheres compared to xenograft derived cells. Sox2, another transcription factor involved in regulating self-renewal pathways, was not expressed in both xenograft derived cells and spheres (Fig. 3B). Immunostaining for stem cell markers, revealed a uniform CD44+, Nestin+, Nanog+, Sox-2-, ABCG2-, TRA-1-60- and CD133- profile for spheroids from xenografted spheres, while cells grown in serum containing medium derived from xenografted spheres consisted of a CD44+, Nestin-, Nanog-, Sox2-, CD133-, TRA-1-60- and ABCG2+ population. The distinct increase in Nanog expression was observed in cells localized within the center of the spheroids (Fig. 4). These results confirmed that prostaspheres are enriched for cancer stem-like cells, which expressed embryonic stem cell markers like Nanog and early progenitor cell markers like Nestin (14).

**Recombinant BC-KLQL-GFP is Selectively Cytotoxic to tumor derived DU-145 Prostaspheres**

Primary tumor derived prostaspheres represent 3D clusters of tumor cells that develop into multicellular globes of fairly large size, display a spatial geometry and closely mimick a tumor. In our previous report, we showed that the PSA activated rNDV was replication restricted for PSA and cytotoxic to CaP cells and prostaspheres (31). As prostaspheres were enriched in stem-like cells, we tested the oncolytic potential of rNDV (BC-KLQL-GFP) on these cell types. Cell line and xenograft derived prostaspheres were
permissive for virus replication (Fig. 5A). Growth kinetics studies with prostate specific antigen (PSA) overlay revealed that virus replication was dependent on the multiplicity of infection (MOI) (Fig. 5B). However, there were no statistical differences in virus yield between the cell line and xenograft derived prostaspheres.

NDV is tumor selective, intrinsically oncolytic and the cytotoxicity against tumor cells is due to multiple caspase dependent pathways of apoptosis (10). BC-KLQL-GFP was selectively cytotoxic to DU-145 prostaspheres and cell death increased proportionally with MOI. At 120 hrs post infection (hpi), with a MOI of 10, the number of viable cells was reduced to approximately 20 percent (Fig. 5C). The percentage cell viability was significantly different between the cell line and xenograft derived prostaspheres at 10 MOI (Fig. 5C). The EC_{50} values were calculated to be 0.11 MOI for cell line derived and 0.14 MOI for sphere xenograft derived prostaspheres in culture. This shows that rNDV is equally effective against stem like or differentiated CaP cells.

**Discussion:**

Although current therapies for prostate cancer eliminate most cells within the bulk of a tumor, it still progresses to androgen independent metastatic disease. According to the cancer stem cell hypothesis, therapy fails because of a small population of (CSCs) that are resistant to conventional treatment (38). It is now widely believed that an effective strategy should target prostate cancer stem-like or tumor initiating cells. The first step will be to identify and enrich for CSCs. The side population (SP) technique was most widely used to identify CSCs in cell culture. However, the impracticality of this method was pointed, as the SP was too small to be reliably detected in long-term prostate cancer cell lines and the cytotoxicity of the Hoechst dye restricted the use of CSCs obtained in
this fashion (39). Several groups have reported isolation of prostate CSCs using stem cell specific markers and in vitro prostasphere culture (4, 29, 32, 35).

In this study, we show that prostaspheres generated from an androgen independent CaP xenograft tumor were enriched in tumor initiating cells. Sphere xenograft derived prostaspheres showed enhanced proliferative and self-renewal capacity when compared to cell line derived spheres. Prostaspheres were also more clonogenic than the parental DU145 cell line. Although $1 \times 10^4$ DU-145 cells did not induce tumors in nude mice, the cell line derived spheres were found to be tumorigenic at the same concentration. DU-145 cells sorted for an ABCG2- population were successful in inducing tumors. It was previously reported that ABCG2 expression identified fast-cycling tumor progenitors, while the ABCG2- population expressed stemness genes and consisted of primitive stem-like cancer cells (30). Staining for several known cancer stem cell markers revealed differences in the staining pattern between DU-145 cells/tumor derived cells and their respective prostaspheres. The overexpression of Nanog and Nestin further confirmed the presence of CSCs in spheres. Nanog, CD44 and Nestin are well-known embryonic stem cell/ progenitor cell markers, which promote cancer stem cell characteristics to CaP cells (14, 19).

Although many reports argue for a CD133 positive population for stemness, DU-145 cells/spheres and xenograft tumor derived cells/spheres were negative for this marker. The anti-CD133 antibody used in this study was against epitope 1 of the glycoprotein and could not have detected other forms. Also, CD133 and CD44 do not always appear to tightly mark a cell population with stem cell characteristics (3, 42). The loss of embryonic stem cell markers such as Nanog and Nestin when cells were grown in
serum containing medium even after a xenograft suggests that stem cells rapidly differentiate in the absence of their niche and factors (15). Loss of the basal differentiation marker CK 14 in prostaspheres indicates the presence of a luminal stem-like/progenitor cell population in DU145. Recently, it was reported that castration resistant prostate cancer cells are stem-like and express a luminal progenitor phenotype (13).

Oncolytic virotherapy (OV) is emerging as an important alternative to conventional cancer treatment modalities. The fact that OVs kill tumor cells by direct and indirect mechanisms of cell death, including apoptosis and by inviting anti-tumor immune response through cytotoxic T cells and NK cells, makes it a worthwhile option to explore them for cancer therapy. The modular nature of the genome, the ability to genetically manipulate, the absence of antibodies in the general population and the extensive safety and efficacy in human subjects makes Newcastle disease virus, an avian paramyxovirus, a promising oncolytic agent (5, 26, 37, 49, 54). Earlier, we showed that NDV could be genetically modified to undergo selective replication in several different types of cancer cells including CaP cells and kill them by intrinsic and extrinsic pathways of apoptosis (10). We have also shown that rNDV defective in interferon antagonism is a safe and effective oncolytic vector (9). Recently, we have genetically engineered NDV to selectively replicate and kill cancer cells that secrete PSA only (31). Although successful Phase I and II clinical trials with naturally occurring strains and preclinical trials in mouse models suggest efficacy for solid tumors, there are no studies that attest NDV’s ability to kill tumor initiating cells. Therefore, we evaluated the PSA mutant rNDV that is selectively cytotoxic to CaP cells for its effect on prostate tumor initiating cells. We
found that the PSA mutant rNDV (31) replicated well in tumor initiating cells from an androgen independent CaP cell line. The virus was also selectively cytotoxic to these cells only in the presence of PSA. PSA activated NDV, could undergo multicycle replication in xenograft derived prostaspheres. Virus yield in these spheres were comparable to that in cell line derived spheres. The rNDV was efficient in inducing cell death of cells/spheres derived from primary xenografts suggesting in vivo efficacy. The EC$_{50}$ (~0.14 MOI) for cytolysis of tumor initiating cells was slightly higher than that was required for the parental cell line, but within the therapeutic margin for safety and efficacy. Our studies suggest that rNDV retargeted to PSA is likely to be beneficial in eradicating CaP tumor initiating and differentiated cells.

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REFERENCE:


cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 65:6207-19.


FIGURE LEGENDS

Table 1. Tumorigenicity and latency of prostaspheres and cells in nude mice. Table shows the different groups of mice that received cell/sphere or xenograft derived cell/sphere injections subcutaneously, the concentration of cells injected, number of mice that developed tumors in each group and latency associated with tumor induction. Nd denotes “not detected”.
<table>
<thead>
<tr>
<th>Tumor transplantation</th>
<th>Cell Type</th>
<th>Concentration</th>
<th>Tumor/total mice</th>
<th>Latency</th>
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</thead>
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<tr>
<td></td>
<td>Cell line</td>
<td>1×10^2</td>
<td>0/10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1×10^4</td>
<td>0/10</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>1×10^6</td>
<td>0/5</td>
<td>ND</td>
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<tr>
<td>Primary xenografts</td>
<td>ABCG2- cells</td>
<td>1×10^2</td>
<td>0/10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1×10^4</td>
<td>2/10</td>
<td>35,50</td>
</tr>
<tr>
<td></td>
<td>Spheres</td>
<td>1×10^2</td>
<td>0/10</td>
<td>ND</td>
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<td>1/10</td>
<td>37</td>
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<td>1/5</td>
<td>30</td>
</tr>
<tr>
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<td>Cells (Sphere xenograft)</td>
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<td>0/3</td>
<td>ND</td>
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<td></td>
<td>Spheres (Sphere xenograft)</td>
<td>1×10^4</td>
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<td></td>
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<td>1×10^6</td>
<td>3/3</td>
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ND: Tumors Not Detectable
Fig.1 Prostaspheres derived from primary tumor xenografts are clonogenic and self-renew. A) Prostaspheres formed from DU-145 cell line 3, 5 and 7 days post induction. B) Proliferation rate of DU-145 ABCG2- cells and sphere xenograft derived prostaspheres for ten serial passages in culture. C) a) DU-145 cell line derived sphere 7 days post induction, b) DU-145 cell tumor derived sphere 7 days post induction, c) DU-145 sphere tumor derived sphere 7 days post induction, d) Soft agar colony 21 days after seeding 1 * 10^3 DU-145 cells, e) 1 * 10^3 DU-145 ABCG2- tumor cells, f) 1 * 10^3 DU-145 sphere tumor cells. D) Clonogenicity of DU-145 monolayer cells, ABCG2- xenograft cells and Sphere xenograft cells expressed as cells seeded per colony formed.
Fig. 2. Immunofluorescence staining for cell types. DU-145 cells, ABCG2- xenograft cells, sphere xenograft cells, cell line derived prostaspheres, ABCG2- xenograft prostaspheres, and sphere xenograft prostaspheres were stained for luminal differentiation marker CK18 and basal differentiation marker CK14.
Fig. 3. **CK14 expression in prostaspheres.** Z-stack imaging was carried out using the Carl Zeiss confocal laser scanning microscope by acquiring in-focus images from selected depths of the prostasphere. Optically sectioned images were saved point to point to analyze the pattern of staining. A) xenograft derived prostaspheres stained for CK14 marker expression, B) Western blot analysis of prostaspheres for stem cell markers Nanog and Sox2. Lane 1: Sphere xenograft derived cells, Lane 2: Sphere xenograft derived prostaspheres.
**Fig. 4. Stem cell markers in DU-145 derived cell types.** Immunofluorescence staining of DU-145 cells, ABCG2- xenograft cells, sphere xenograft cells, cell line derived prostaspheres, ABCG2- xenograft prostaspheres, and sphere xenograft prostaspheres for stem cell markers such as CD44, Nestin, Nanog, ABCG2 and TRA-1-60.
Fig. 5. Recombinant PSA mutant NDV replicates and effectively kills CaP tumor initiating cells. A) BC-KLQL-GFP infection in DU-145 sphere xenograft prostaspheres at 24 and 72 hours post infection. B) Virus yield in DU-145 cell line derived, ABCG2- and sphere xenograft derived prostaspheres at multiplicity of infection (MOI) 0.01 0.1, 1 and 10. C) Percentage cell viability of DU-145 cell line, ABCG2- and Sphere xenograft derived prostaspheres at 120 hours post-infection with BC-KLQL-GFP using 0.01, 0.1, 1 and 10 MOI. Percentage cell viability at MOI 10 was significantly different between cell line and xenograft derived prostaspheres (p< 0.0001).
CHAPTER-4

General Conclusion

In this study, we explored tumor specific protease targeting of Newcastle disease virus (NDV), for enhanced spread and oncolysis. We developed a recombinant NDV (rNDV) specifically targeted to prostate cancer, by genetically modifying the virus to replicate only in the presence of prostate specific antigen (PSA). We altered the cleavage site motif of the viral fusion protein to that of PSA so that active fusion peptide could be produced only in the presence of active PSA. PSA activated virus failed to replicate in the absence of PSA and the availability of active PSA directly correlated to the virus yield in prostate cancer cells. The engineered PSA-activation dependent NDV is likely to be devoid of off-targeting issues generally encountered in phase I clinical trials with naturally occurring strains of NDV. Besides, the enhanced fusogenicity in the presence of active PSA in androgen responsive cells, allowed for increased spread and cytotoxicity.

Further, we showed that prostaspheres generated from an androgen independent DU-145 CaP xenograft tumor were enriched in tumor initiating cells and found that the PSA mutant virus replicated well in these tumor initiating cells. The virus was also selectively cytotoxic to sphere cells only in the presence of PSA. PSA activated NDV, could undergo multicycle replication in xenograft derived prostaspheres and virus yield in these spheres were comparable to that in cell line derived spheres. The rNDV was efficient in inducing cell death of cells/spheres derived from primary xenografts suggesting in vivo efficacy. The EC50 (~0.14 MOI) for cytolysis of tumor initiating cells was slightly higher than that was required for the parental cell line (0.11 MOI), but within
the therapeutic margin for safety and efficacy. Our studies suggest that rNDV retargeted to PSA is likely to be beneficial in eradicating CaP tumor initiating cells and the excellent \textit{in vitro} data poise it for immediate preclinical and Phase I/II clinical trials. The results highlight the oncolytic ability of engineered NDV against prostate cancer. The recombinant virus will radically improve the way in which prostate cancer is treated at present.